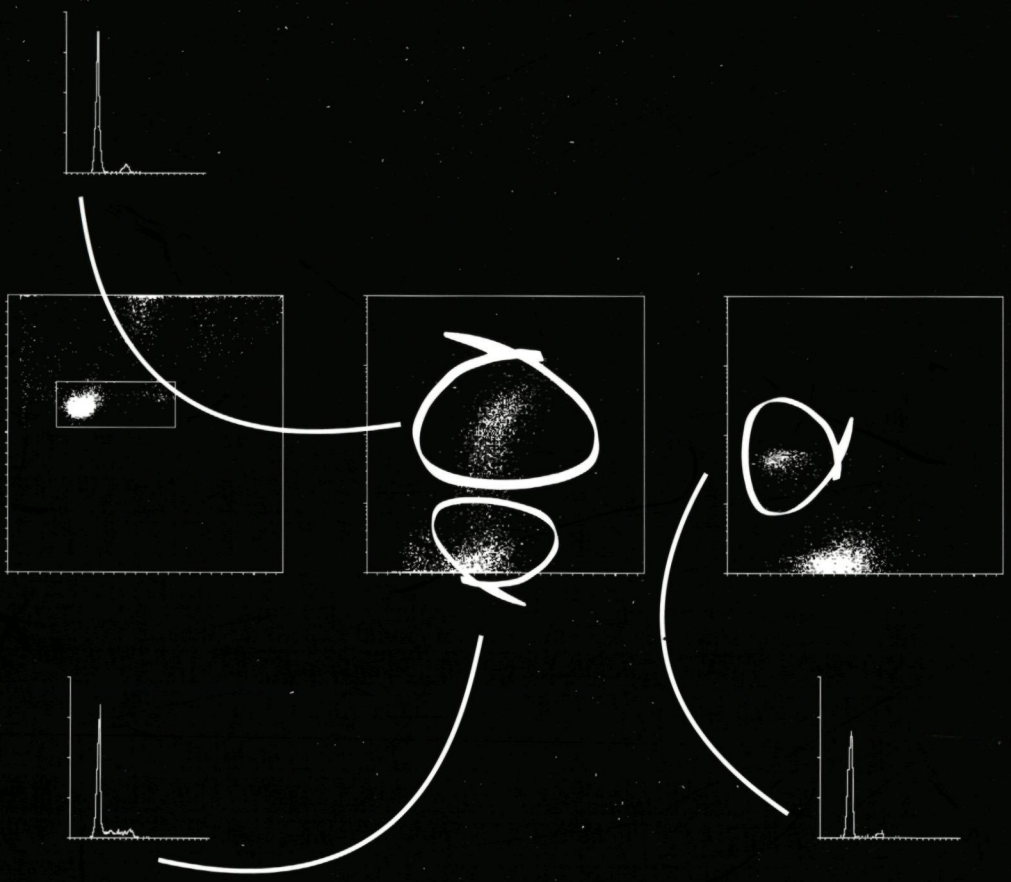
Multiparameter flow cytometry as a tool to study psoriasis



Conrad P. Glade

MULTIPARAMETER FLOW CYTOMETRY AS A TOOL TO STUDY PSORIASIS

MULTIPARAMETER FLOW CYTOMETRY AS A TOOL TO STUDY PSORIASIS

Een wetenschappelijke proeve op het gebied
van de Medische wetenschappen

Proefschrift ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op
maandag 9 juni 1997
des namiddags te 3.30 uur precies

door

Conrad Peter Glade
geboren op 17 november 1963 te Heidelberg

Promotor:	Prof. Dr. Dr. P.C.M. van de Kerkhof
Co-promotores:	Dr. P.E.J. van Erp Dr. E.M.G.J. de Jong
Manuscriptcommissie:	Prof. Dr. T.J.M. de Witte Prof. Dr. J. Schalken Dr. A. Hanselaar

ISBN 90-9010652-9

Financial support for printing this thesis was kindly provided by ConvaTec B.V., Coulter/Immunotech Nederland, Galderma Nederland B.V., Glaxo Wellcome B.V., Hermal A.G., Janssen-Cilag B.V., LEO Pharmaceutical Products B.V., Novartis Pharma B.V., Roche Nederland B.V., Schering A.G. (Center of Dermatology), N.V. Varitex and Yamanouchi Pharma B.V.

Individualitat ist das, was mich von der Welt absondert, Liebe das, was mich mit Ihr verbindet. Je starker die Individualitat, desto stärker erfordert sie Liebe

Walther Rathenau (1867-1922)

CONTENTS

Chapter 1

GENERAL INTRODUCTION

1.1 General aspects of psoriasis

EPIDEMIOLOGY, PATHOGENESIS AND MORPHOLOGY

Psoriasis is a frequently occurring chronic skin disorder with remissions and exacerbations. Approximately 2% of worlds population is affected. In general, the disease manifests itself at adolescence. Relatively seldom, especially following a focal infection, the disease may occur in children. In 5-10% of the patients with psoriasis involvement of the joints occurs. Although the pathogenesis of psoriasis remains unclear, genetic and environmental factors are causative: psoriasis is supposed to have a polygenic inheritance. Environmental factors comprise systemic influences such as focal infections, psychological stress and medication, for instance β sympaticolytic drugs, lithium carbonate and antimalarial drugs. But also topical factors may elicit a psoriatic lesion such as trauma of the skin, skin disorders or chemical and physical injuries. It is an attractive experimental approach to study the effect of trauma, UVB and application of chemical substances on normal skin in order to understand early pathogenetic events.

Figure 1. Psoriatic plaque located on the elbow.

The clinical appearance of psoriasis characterized by sharply demarcated erythematous plaques (Figure 1) with a symmetrical distribution. Most patients present with this "chronic plaque-type" psoriasis. However, in children, an exanthematic distribution pattern of small lesions occurs: "psoriasis guttata". In case of severe psoriasis the whole bodysurface may be affected resulting in a "erythroderma". Sometimes sterile pustules may complicate the manifestation: "pustular psoriasis". Lesions may occur at every part of the body, although the face, in general, is spared. Predilection sites are the extensor surfaces of elbows and knees, the sacral region and the scalp. The nails may exhibit pitting, distal onycholysis (Figure 2) and subungual keratosis. The joints may show the picture of psoriatic arthropathy, characterized by a seronegative arthritis with a typical radiographic pattern. The extent and the expression of psoriasis differs from patient to patient. Also within the same patient the expression pattern can differ considerably.

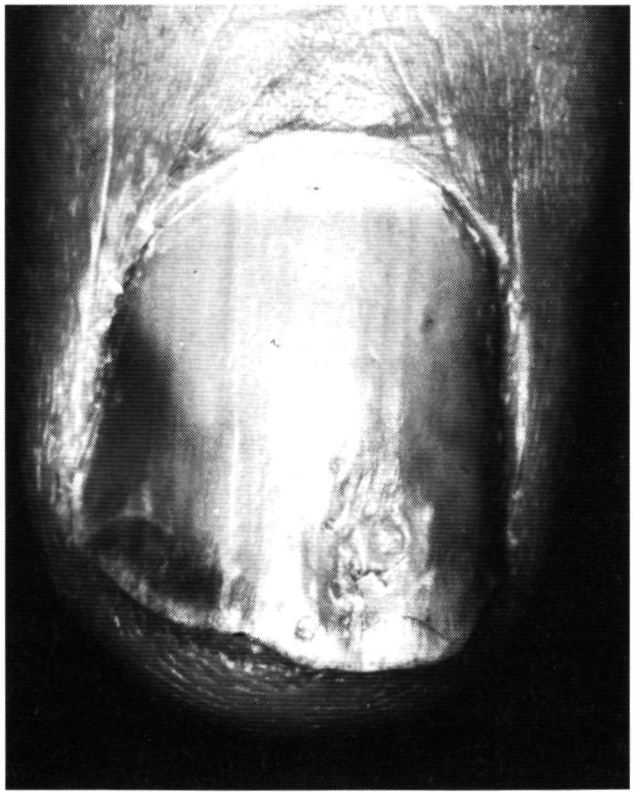


Figure 2. Psoriasis unguium with distal onycholysis and pitting.

The clinical characteristics of the psoriatic lesion are a reflection of cutaneous inflammation, epidermal hyperproliferation and disturbed keratinization. In case of a severe dominance of inflammation pustule formation is prominent, in case abnormal differentiation and epidermal proliferation dominate, excessive scale formation might be expected. However, so far no studies are available which conclusively demonstrate the correlation between clinical and cell biological features. Also the causes of these key processes are unknown so far.

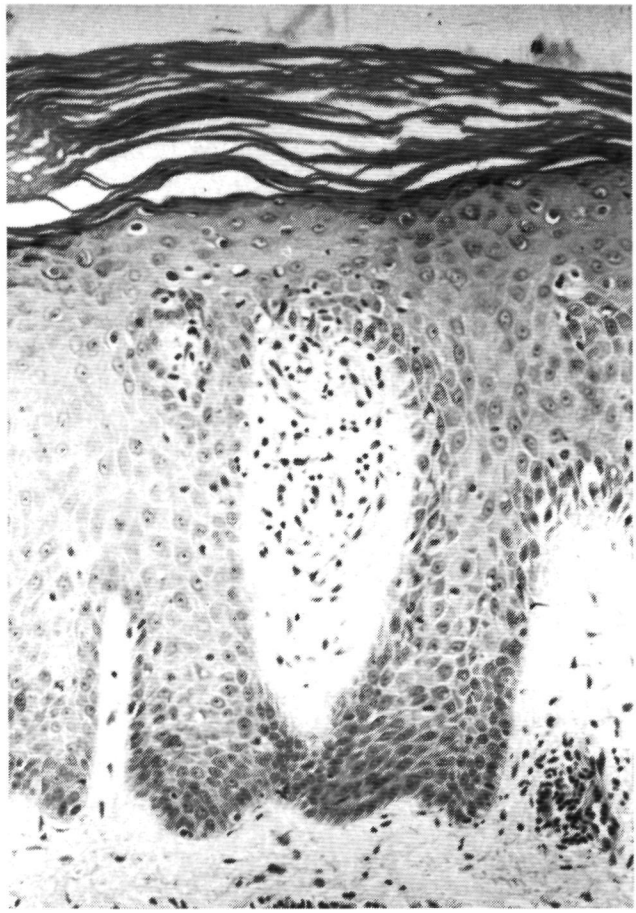


Figure 3. Histologic section showing typical features of psoriasis: epidermal acanthosis, focal parakeratosis, elongated rete ridges and a mixed inflammatory infiltrate.

The histopathological features of psoriasis consist of epidermal acanthosis, thinning of the suprapapillary plate and infiltrate formation in dermis and epidermis. The stratum corneum is characterized by parakeratosis, a focal arrangement of DNA containing corneocytes without underlying stratum granulosum. Spongiform micropustules of Kogoj in the epidermis and microabscesses of Munro in the stratum corneum are observed to be filled with polymorphonuclear leukocytes end remnants of these cells. Figure 3 and 4 provide some illustration. The highest inflammatory activity of polymorphonuclear leukocytes is observed in pustular psoriasis. In exacerbating plaque-type psoriasis, the number of infiltrating polymorphonuclear leukocytes increases with the progression of lesions. However, a reliable quantitative approach to assess the composition of the epidermal infiltrate has to be developed.

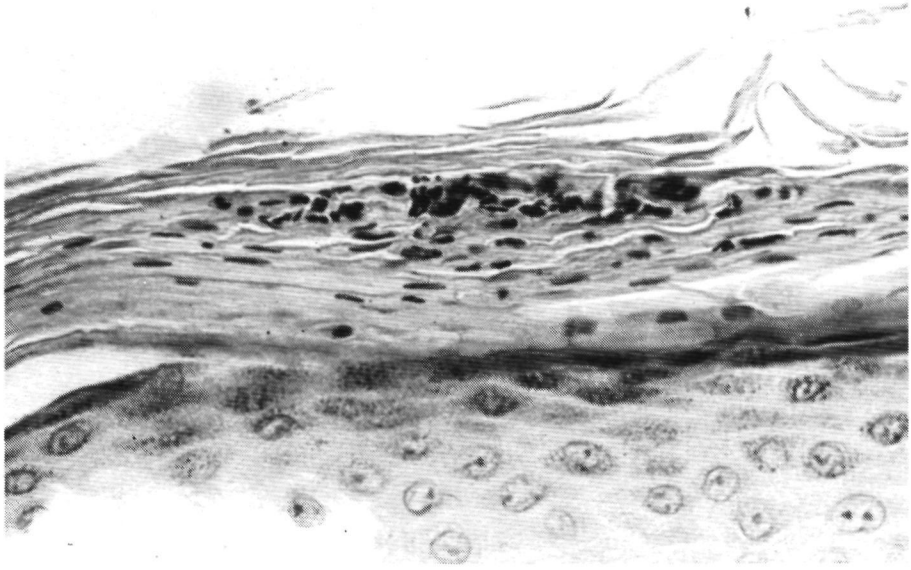


Figure 4. Detail of the psoriatic epidermis showing a microabsces of Munro containing polymorphonuclear neutrophils.

THERAPY

There is at present no cure for psoriasis, only suppressive therapy. Therapeutic management of psoriasis requires individualisation. Many different topical and systemic antipsoriatic treatments are available. The treatment of choice depends upon type, extent, and duration of psoriasis, symptoms of the lesions, age, sex, general health and concomitant medication. Also social factors (occupation and travel distance) and psychological factors (personality, acceptance, motivation) play an important role. Different treatment modalities may be combined. In this way, side-effects are minimised while the clinical efficacy is maintained or even improved. For a detailed review on the treatment of psoriasis the reader is referred to the article of Greaves et al[1].

Topical treatment

Emollients

The application of emollients hydrates and softens the scaly and hyperkeratotic surface of psoriatic plaques. In about 35% of patients twice-daily application resulted in a reduction of itching, pain and of clinical severity and extension. Greasy emollients are less accepted by patients.

Keratolytic agents

Salicylic acid (2-20% in ointment) is a widely used keratolytic agent. It can be combined with coal tar, dithranol or corticosteroids, and then increases the absorption of these agents. Applications of high concentrations on large body areas should be avoided because of the risk of intoxication.

Coal tar

In the treatment of psoriasis coal tar is often combined with ultraviolet B phototherapy. Coal tar preparations are frequently used in shampoo preparations. Coal tar therapy is safe and usually well-tolerated. Limitations are the unpleasant odour and the irritative potential. Contra-indications are erythrodermia and generalized pustular psoriasis.

Dithranol

The formation of free radicals by dithranol is responsible for the antipsoriatic activity. However, these radicals are also responsible for the irritation, especially of the perilesional skin. Dithranol is oxidized to coloured products that stain the skin and

clothes brown or purple These side-effects are partly avoided in the so-called "short-contact regimen" in which the application times of dithranol are between 10 and 60 minutes Dithranol therapy is very safe

Corticosteroids

Topical corticosteroids are the most frequently used therapy against psoriasis Advantages are the short-term efficacy, absence of odour and staining, and relatively low cost Local side effects consist of thinning of the skin, striae, hypopigmentation, teleangiectases, allergic contact dermatitis and tolerance (tachyphylaxis) Withdrawal of potent topical corticosteroids may induce a serious relapse, resulting in a generalized pustular psoriasis If applied on large body areas topical corticosteroids can cause suppression of the pituitary-adrenal axis

Vitamin D_3 analogues

Calcipotriol is a vitamin D_3 analogue that can be applied topically Calcipotriol 50µg/g twice daily is indicated in mild-to-moderate psoriasis Up to 100g can be applied weekly without significant influence on calcium metabolism No systemic side-effects have been observed Local irritation of the skin is reported in 10-25% of patients However, this irritation is usually transient

Photo(chemo)therapy

Ultraviolet radiation (UVR) is effective in the treatment of psoriasis Phototherapy with ultraviolet B (290-320nm) can be used as monotherapy or in combination with for instance tar and dithranol Mildly erythematogenic dosages of UVB are given three times weekly Side effects are increased ageing of the skin and cutaneous malignancies Photochemotherapy combines the photosensitizing drug methoxysalen with ultraviolet A (PUVA) in the range of 320-400nm PUVA is a highly effective treatment Short-term adverse events are burning, nausea, and pruritus in 10-20% of patients The long-term problems consist of photodamage to the skin, pigmentation, lentigines and cancer related to the exposure to UVA Seldom cataract and hepatotoxicity have been reported PUVA can be indicated in severe psoriasis vulgaris, generalized pustular psoriasis and palmoplantar pustular psoriasis

Systemic treatment

Corticosteroids

Oral corticosteroids should be reserved for patients with severe erythrodermic or generalized pustular psoriasis if other drugs are contraindicated. Prolonged use leads to Cushing's syndrome and suppression of the adrenal-pituitary axis. After withdrawal a significant risk for the development of a therapy-resistant pustular psoriasis exists.

Methotrexate

The folic acid antagonist methotrexate (MTX) blocks cycling cells in G_1 phase, resulting in a reduction of the number of cycling cells. All rapidly cycling tissues are affected including the gastrointestinal and the germinative epithelium. Methotrexate also has an immunosuppressive effect. Indications are severe psoriasis vulgaris, erythrodermic psoriasis, generalized pustular psoriasis, severe psoriatic arthritis and severe nail psoriasis. Side-effects of MTX are anorexia, nausea, hair-loss and haematological abnormalities. Interstitial pneumonitis is a rare side-effect. Furthermore, MTX may be mutagenic. Long-term treatment may cause liver fibrosis or cirrhosis. Therefore after 1.5 g MTX (cumulative dose) a liver biopsy is indicated. Laboratory investigations should be performed at regular intervals (4-6 weeks). Contraindications are pregnancy or pregnancy wish (males and females), disturbed renal or hepatic function, gastric ulcer, alcohol abuse. Patient compliance should be high. The maximum weekly dose is 15 mg/week, usually administered in 3 oral doses at 12-hour intervals (Weinstein-schedule).

Retinoids

In the treatment of psoriasis retinoids have antiproliferative and anti-inflammatory effects. Furthermore, a normalization of disturbed keratinocyte differentiation occurs. Important indications are severe pustular or erythrodermic psoriasis. Retinoids can be combined with other treatments. Side-effects are: dryness of eyes, nasal and oral mucosa, cheilitis, xerosis, pruritus, palmoplantar desquamation, hair loss, elevation of cholesterol, triglyceride and elevation of liver enzymes. During long-term treatment hyperostosis of the spine may be observed. However, the causal relationship between spinal abnormalities retinoids is discussed[2]. and As retinoids are teratogenic, adequate anticonceptive measures should be taken until 2 years post-treatment. Contra-indications are pregnancy or pregnancy wish, hepatic dysfunction, insufficient patient compliance and strongly elevated serum levels of cholesterol or triglycerides. Regular

clinical and laboratory examinations are indicated. Before treatment an X ray of the spine should be made.

Cyclosporin

This relatively new drug inhibits the CD4+ T lymphocyte function. Cyclosporin is indicated in refractory psoriasis. Neprotoxicity and the development of hypertension are serious side-effects. The lowest possible dose should be given. Other side-effects are gingival hyperplasia, hypertrichosis, headache, nausea and diarrhoea. Contra-indications are impaired renal function, hypertension, immunodefficiency, malignancies, pregnancy, infections and epileptic disorders. The use of concomitant nephrotoxic, cytotoxic, immunosuppressive treatment or radiation therapy should be avoided. Frequent clinical and laboratory analysis is indicated during treatment.

Combined therapy

As discussed before, a large scala of antipsoriatic approaches exist. Each approach with its own advantages and limitations. Combination therapy has the important advantage that clinical efficacy is maintained or even improved, while side-effects are reduced. Examples of rational combinations are: tar and UVB, dithranol and UVB, and retinoids and PUVA (Re-PUVA). Often treatment with MTX, retinoids or cyclosporin is combined with topical application of corticosteroids or calcipotriol. Systematic search for the most optimal combinations are necessary.

Development of new treatment modalities

An important aspect in antipsoriatic drug development is the realization of long-term management. The cumulative therapeutic potential has to be high. For example long-term use of topical corticosteroids is impeded by their side-effects. Therefor new antipsoriatic approaches are needed.

Various new antipsoriatic agents are in clinical investigation. Included are new vitamin D_3 analogues, new retinoids and new imunomodulators. Hydrocolloid occlusion as a monotherapy is effective in the treatment of psoriasis. In combination with topical therapy high efficacy can be obtained. New combinations of these therapies could mean an important progress in long-term antipsoriatic treatment. In this respect it is of importance to study the effect of promising compounds on the acute phase of the psoriatic process. New models to induce these initial changes are required.

To be able to compare therapy efficacy and to analyze *in vivo* models for psoriasis a quantitative method is required In the next chapters we discuss this issue

MEASUREMENT OF SEVERITY OF PSORIASIS

Clinical assessment

Erythema, induration, desquamation and bodysurface

The pathologic disturbance of the skin in psoriasis results in macroscopic changes clearly visible for the naked eye The psoriatic plaque is characterized by redness (erythema), thickening (induration) and scaling (desquamation) The clinical assessment of psoriatic lesions is based upon classification of these signs into a 5-points scale An advantage of this method is that no instrumentation is needed, resulting in easy and time-saving assessment However, a few disadvantages exist Firstly this kind of assessment is not quantitative, but rather subjective Secondly, a significant interobserver variability may be present Furthermore, the clinical signs that are estimated may not be a direct reflection of the pathogenetic processes in psoriasis Assessment of the percentage bodysurface that is affected by psoriasis is difficult The human eye is notoriously poor as an estimator of area fraction[3] It has been shown that a considerable variability exists between observers[4] In the frequently used Psoriasis Area and Severity Index (PASI), described by Fredrikson and Petterson[5], both the percentage of involved body surface and the degree of erythema, induration and desquamation are estimated in four body areas Then, using a formula, a value between 0 and 72 can be calculated This integral approach to assess the severity of psoriasis is broadly accepted, but also discussed for the above-mentioned reasons[6,7]

Instrumental assessment

Obviously, further development of methods to measure psoriasis severity is needed These methods have to fulfil the following conditions

(i) **to quantify the response to therapy**

(ii) **to eliminate the interobserver variability**

(iii) **to approach the cell biological reality**

Non-invasive techniques

Many investigators have developed and applied non-invasive biophysical techniques to quantify and monitor psoriasis (image analysis, measurement of transepidermal water loss, ultrasound, blood flow, electric techniques). Attempts have been made to quantify the size of lesions by image analysis[3]. A videomicroscopic study has been performed on treatment of psoriasis with 1α,24 dihydroxyvitamin D_3[8]. Measurement of transepidermal water loss (TEWL) provides information on the barrier function of the skin. In psoriasis TEWL is increased. Improvement of psoriatic plaques by topical therapy has been evaluated by measurement of TEWL[9,10]. Also ultrasound imaging of psoriatic skin has been used as technique to evaluate treatment of psoriasis[11]. It was shown that skin thickness is significantly increased in psoriatic skin compared to normal skin. Furthermore, a differentiation between epidermal and dermal changes was possible. By using laser Doppler velocimetry (LDV) it has been shown that skin blood flow is increased in psoriatic lesions[12]. Other investigators measured blood flow after antipsoriatic therapy[13-15] and in the active edge of psoriatic lesions[16,17]. An other non-invasive technique consists of impedance measurement. The electrical level is decreased in psoriasis and returns to normal after different antipsoriatic therapies[18,19].

By these non-invasive bioengineering techniques certain biologic information on psoriasis and its treatment are obtained. In general, the efforts for patient and clinician are moderate. However, most data deal with descriptive phenomena. This means that the third condition for quantitative assessment is not fulfilled. To obtain more specific information, the use of invasive techniques seems unavoidable.

Invasive techniques

Invasive procedures imply that biopsies are taken from volunteering subjects. Daily monitoring of psoriatic lesions is therefore practically impossible. However, an advantage over non-invasive procedures is that not only epiphenomena are studied: invasive techniques permits access to the pathologic process itself.

To study changes at the microlevel, immunohistochemistry is the well-established approach. Cell biological information is obtained by using monoclonal antibodies against certain markers. The following markers are regularly used at the Department of Dermatology of the University Hospital Nijmegen[20-22]: Ki-67 and MIB1 are directed against a nuclear antigen present in cycling epidermal cells. Markers for epidermal differentiation are filaggrin, involucrin, transglutaminase, keratin 10 (recognized by RKSE60) and keratin 16 (recognized by Ks8.12). Information on inflammatory and

immunological cells can be obtained by markers against PMNs (elastase), T-lymphocytes (T11), monocytes (WT14) and Langerhans cells (OKT6). All these mesenchymal cells express the intermediate filament protein vimentin.

In the present thesis, the changes at the microlevel have been quantified with a relatively new method in dermatology for psoriasis research and treatment evaluation: multiparameter flow cytometry. Flow cytometry is a technique in which physical or/and chemical characteristics of cells (or other particles) are quantified for scientific, diagnostic or therapeutic purposes. Single cells pass through a fluid stream in the measuring apparatus and different markers can be quantified. This technique is very well applicable on epidermal single cell suspensions prepared from normal or psoriatic skin[23]. However, it should be realized that no information is given on the histologic topography.

The common elements in all flow cytometers are: a light source, an optical bench to focus and direct that light, a liquid stream containing particles flowing through the focused light beam, photodetectors and amplifiers to measure and record the intensity of light signals and a computer system to analyze these lights signals. The principle of flow cytometry is summarized in Figure 5: the particles (i.e. cells, viruses, bacteria, fungi, organelles, chromosomes, liposomes) flow rapidly in an aqueous solution and pass one or more laser beams at a rate of several hundreds of particles per second. The particles are stained by fluorescent dyes, which emit light after excitation by the laser. In optimal conditions each particle passes the laser light separately and causes scattering of the light (defined by shape, size and granularity of the particle) and/or generation of specific fluorescence signals depending on the fluorochromes used to stain the particles. Signals of different wave lengths are produced in this way, providing quantitative information on different cell parameters. Optical filters are used to separate the different emitted wave lengths. The signals are measured by photodetectors, amplified and stored in a computer for further evaluation.

In contrast to immunohistochemistry, flow cytometry provides quantitative information of cell characteristics. As a great quantity of cells is measured, the statistical reliability is high. Furthermore, flow cytometry permits simultaneous measurement of different cell parameters (multiparameter flow cytometry). Flow cytometry is a powerful and promising approach to fill the niche for quantitative cell biological technology assessment. Therefore, this technique was selected for the studies in the present thesis. In the next chapter a short review on the history of flow cytometry will be given and applications in dermatology will be introduced.

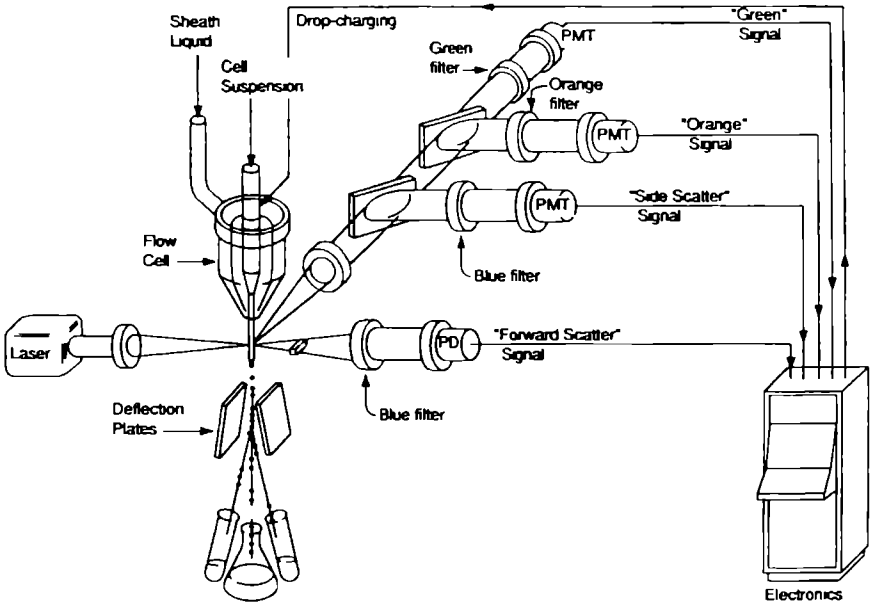


Figure 5. Schematic drawing of a flow cytometer

1.2 Flow cytometry

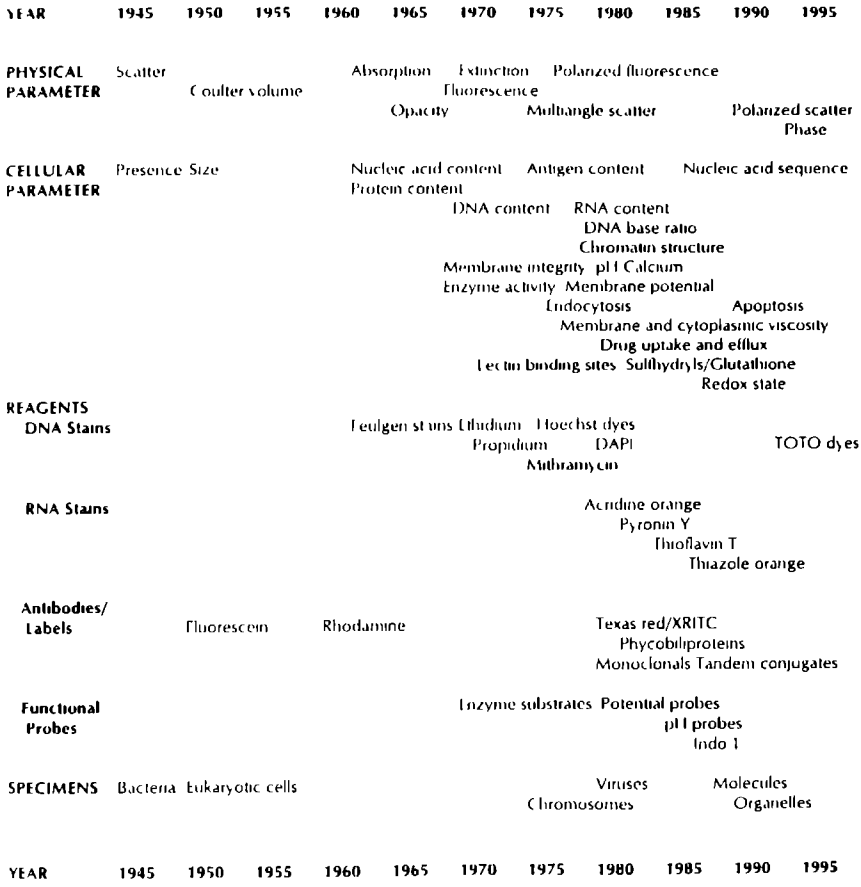
HISTORY

The first description of flow cytometry was published in *Science* in 1934 by Moldavan[24]. He introduced the concept of counting cells (blood cells) flowing through a capillary tube, by using a photoelectric sensor. In the 1940's fluorescence microscopy began to be used in conjunction with fluorescent stains for nucleic acids in order to detect malignant cells[25]. Antibody technology enabled wider use of fluorescent stains[26]. The basic principles of modern flow cytometry consist of a synthesis of microscopy, blood cell counting instruments and the ink jet technology, which was developed in the 1960's for computer printers. In 1969 a microscope-based system was described whereby histograms could be generated based on ethidium bromide fluorescence of alcohol-fixed cells[27]. Until then, counting of small particles flowing through a tube was not successful, because of turbulence in the fluid stream. The solution for this practical problem was to apply the principles of laminar flow in the flow system. The suspension of particles was injected into the centre of a faster flowing stream. In this way turbulence was avoided and reliable counting enabled.

In 1970 Kamentsky began to produce flow cytometers with helium-neon or argon ion laser light sources[28]. The flow cytometer using the argon ion laser was to some extent suitable for analysis of DNA content. In 1972 Herzenberg improved his Fluorescence Activated Cell Sorter (FACS). This machine (argon ion laser) could detect relatively weak fluorescence of cells stained with fluorescein or rhodamine[29]. The development of dual-beam or multibeam flow cytometers enabled three- or four- (or more) parameter measurement. This involved a major escalation in cost as adequate microprocessors had to be developed.

Various fluorescent dyes became available. Flow cytometry was used successfully in the analysis both of cellular DNA content, RNA content and of functional cellular parameters. In Table 1 a brief outline of flow cytometric history from 1944 to 1994 is given. In the field of molecular biology and molecular genetics flow cytometry and sorting proved its value. Important progress was made in the clinical use of fluorescence flow cytometry. In oncology DNA content analysis remains the most widely used flow cytometric procedure. Other important applications are the automatic performance of the differential leukocyte count and of the blood reticulocyte count, the immunophenotyping of human leukaemias and the determination of the relative and absolute number of CD4-positive T-cells (HIV-infection).

Table 1. Flow cytometric history 1944-1994



In dermatology the normal skin and the pathologically changed skin have been extensively studied using flow cytometric techniques. The application of flow cytometry in psoriasis-research is discussed in the next chapter. Research on premalignant and malignant dermatoses, such as Bowen's disease, actinic keratosis, keratoacanthoma, basal cell carcinoma and squamous cell carcinoma, is especially focused on ploidy determination. Clinical features as disease progression, local invasiveness and metastatic behaviour are possibly correlated with aneuploidy in lesions[30]. So far, however, this single-parameter DNA content analysis has not been successful in distinguishing keratoacanthoma from squamous cell carcinoma[31].

Simultaneous measurement of differentiation markers might be of importance in this regard. Flow cytometric measurement of DNA-ploidy in dysplastic nevi, congenital nevi and melanomas has been explored as test for melanoma diagnosis and prognosis[32-36]. Aneuploidy has also been detected in clinically benign nevi. This may implicate that flow cytometry can detect early premalignant changes which do not manifest themselves morphologically. Improved aneuploidy measurement in melanomas can be reached by multiparameter flow cytometry by identification and exclusion of diploid non-tumour cells[37]. Fundamental research of immunological dermatoses has benefited considerably from flow cytometry. Also in pathogenesis-oriented diagnosis and monitoring of disease activity flow cytometry proved its value[38].

FLOW CYTOMETRY IN PSORIASIS

The epidermis consists of different epidermal subpopulations. The majority of cells are keratinocytes which express keratin as their intermediate filament. All proliferating keratinocytes participate in the cell cycle (Figure 1).

Four different phases can be recognized. Firstly, the S-phase in which cells replicate their DNA. The DNA content increases from diploid (2C) to tetraploid (4C). The interval between DNA-synthesis and the next mitosis is referred to as G_2-phase (G=gap). The cell division phase is called the M-phase (M=mitosis). Cells in G_1-phase are recovering from mitosis or preparing (biosynthesis of enzymes) to enter another cell cycle. Probably at a point in early G_1-phase cells (decision point) "choose" whether they remain in the cell cyclus (DNA-synthesis), or escape from it by committing themselves to terminal differentiation or by entering a quiescent state (G_0-phase). Figure 2 shows a histogram that represents the DNA distribution of an epidermal cell population. Obviously, in hyperproliferative epidermis a relatively large amount of keratinocytes will be present with more than diploid cellular DNA content. These keratinocytes are in S-, G_2- and M-phase.

The basal keratinocytes can be distinguished by their ability to express the keratin pair K5 (58 kD) and K14 (50 kD). As they commit to terminal differentiation, they leave the cell cycle, leave the basal layer and begin their migration towards the surface of the skin. The keratinocytes then enter the spinous layer and are characterized by a shift in keratin synthesis from the K5/14 pair to the K1/K10 pair. Later in the maturation

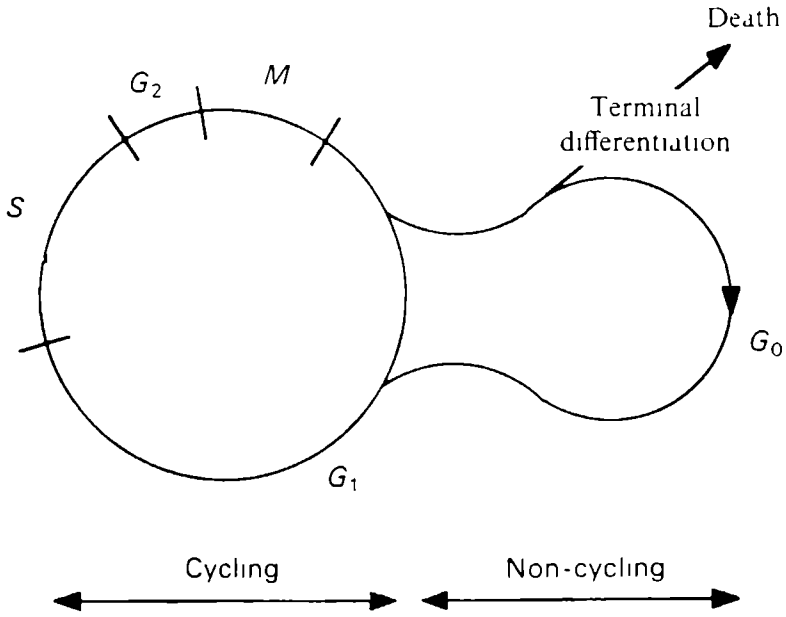


Figure 1. A diagram of the cell cycle

process keratinocytes synthesize involucrin and filaggrin and activate epidermal transglutaminase. Some monoclonal antibodies that react with basal and suprabasal epidermal cells are listed in Table 2.

The first flow cytometric studies on psoriasis consisted of single-parameter measurement of DNA content. Nuclei of epidermal cells were stained with fluorescent dyes that quantitatively conjugate with DNA. Frequently used fluorochromes were ethidium bromide, introduced in 1967[39], and propidium iodide (1969)[40]. Both dyes selectively intercalate with the double-stranded regions of nucleic acids. As they also react with RNA, reliable DNA content analysis requires pre-treatment with RNase. Techniques to analyse the proliferative activity of normal and abnormal keratinocytes were developed and improved[41-44]. It was shown that significantly higher numbers of cycling cells are present in epidermis from involved psoriatic skin compared to normal skin[45,46]. Special emphasis was put on the preparation of epidermal cell suspensions[47,48]. A first flow cytometric evaluation of wellknown antipsoriatic therapies was carried out[49].

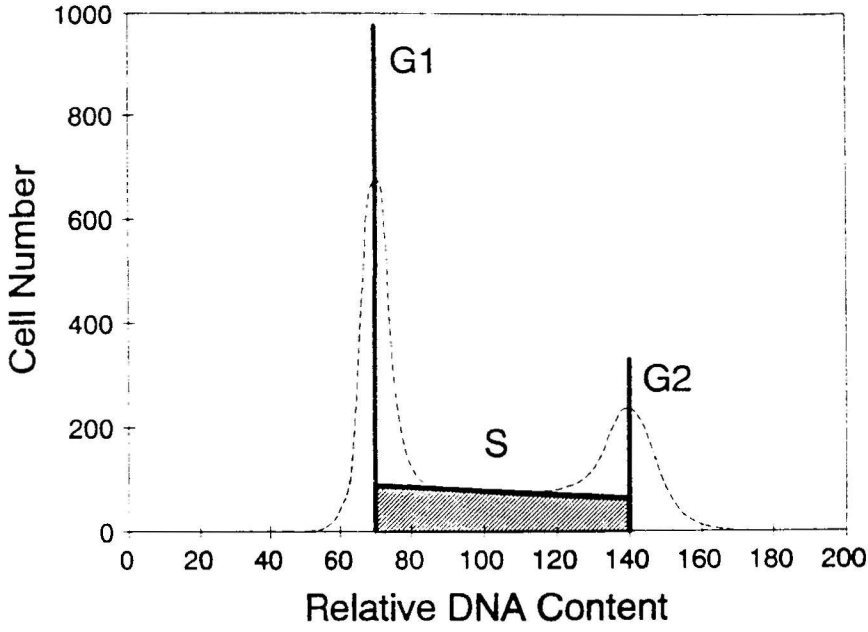


Figure 2. DNA histogram from epidermal keratinocytes. The peak corresponds with cells in G_0/G_1-phase.

Table 2. Monoclonal antibodies reactive with epidermis

Monoclonal antibody	Directed against	Source
RCK105	Keratin 5 and 8	Dept. Mol. Biol., Maastricht
RKSE60	Keratin 10	Dept. Mol. Biol., Maastricht
DE-K10	Keratin 10	ICN
K14	Keratin 14	Sigma
LL002	Keratin 14	Novocastra
Ks8.12	Keratin 13 and 16	Sigma
LL024	Keratin 16	Novocastra
Anti-filaggrin	Filaggrin	BTI
Anti-transglutaminase	Transglutaminase	BTI
Anti-SKALP	SKALP	Dept. Dermatology, Nijmegen
Anti-involucrin	MON 150	Dept. Dermatology, Nijmegen
Ki-67	Proliferating cells	Dakopatts
MIB-1	Proliferating cells	Immunotech
Anti-PCNA	PCNA	Boehringer
Anti-BrUrd	Proliferating cells	Dakopatts

From a variety of studies it was concluded that psoriatic hyperproliferation results from reduced turnover time and cell cycle time (T_c) compared to normal skin[50]. The average T_c for normal skin was calculated to be about 209 hours, whereas in involved psoriatic epidermis T_c was significantly lower reaching values of 36 hours[51]. However, evidence against this classic assumption is cumulating: a recent study by Van Erp *et al.* using flow cytometry in combination with IdUrd labelling showed that there seems to exist no major difference between normal and hyperproliferative skin with respect to cell cycle time and duration of individual cell cycle phases[52]. This *in vivo* study strongly suggests an increased growth fraction to be a sufficient case for the permanent increase of the germinative population in psoriasis, as computer simulations were already indicating in 1979[53].

It is important to realize that by using single-parameter flow cytometry no reliable discrimination between different epidermal subpopulations can be reached. Therefore values for proliferative activity reflect the overall epidermal proliferative activity, including all epidermal cells, i.e. basal keratinocytes, suprabasal keratinocytes and nonkeratinocytes. Progress in this field was made by the development of double-labelling procedures. Different investigators performed simultaneous examination of epidermal proliferation and keratin expression in normal and psoriatic skin[23,54-56]. It was shown that the number of basal cells (keratin 5/14) was increased in psoriatic epidermis compared to normal epidermis. The number of keratin 10-positive cells was decreased in psoriatic lesions. Proliferating cells were especially located in the basal layer. The intermediate filament protein vimentin was used to discriminate between keratinocytes and non-keratinocytes[23,55,57].

At the Department of Dermatology in Nijmegen an Epics Elite flow cytometer is available for flow cytometric research (see Figure 3). During the investigations described in the present thesis two lasers were present in the apparatus: an air-cooled 488 nm Argon laser and 633 nm HeNe laser. This means that excitation is possible at two different wavelengths permitting the measurement of multiple signals. Suitable fluorochromes for double-labelling procedures with the Argon laser are propidium iodide (DNA stain) and fluorescein which both absorb light at 488 nm and have different emission spectra: peak values of 630 nm (red light) and 525 nm (green light), respectively. Phycoerythrin is also excitated at 488 nm. Addition of the relatively cheap HeNe laser offers the possibility to introduce new fluorochromes, which are excitated by the 633 nm-signal. New possibilities for three-colour cytometry are within reach.

Figure 3. The Coulter Epics Elite flow cytometer

1.3 Aims of the thesis

METHODOLOGY

Psoriasis is a chronic disease with epidermal hyperproliferation, impaired keratinization and cutaneous inflammation. These cell biological features are clinically expressed as induration, scaling and erythema of the psoriatic lesion. In clinical assessment of disease activity the scores for induration, scaling and erythema are estimated on a 5-points scale, resulting in a numerical value. This clinical scoring, however, is subjective and introduces inaccuracy.

In the evaluation of antipsoriatic therapies the quantitative assessment of disease activity is essential. Flow cytometry permits simultaneous, quantitative measurement of different epidermal cell parameters. This approach might be an alternative for clinical scoring. Therefore the first aim of the present thesis is:

(1) TO DEVELOP AND VALIDATE A NEW APPROACH TO MONITOR DISEASE ACTIVITY.

This new method should permit quantitative measurement and reflect the key processes in the pathogenesis of psoriasis. Validation of this method should comprise correlation analysis with clinical parameters.

IN VIVO MODELS

Several *in vivo* models for psoriasis exist. These models can be used to study the dynamics of epidermal proliferation, keratinization and inflammation. Quantitative analysis might reveal aspects of the pathogenesis of psoriasis. *In vivo* models might allow standardized comparison of different antipsoriatic therapies. Therefore the second aim is:

(2) TO CHARACTERIZE POTENTIAL *IN VIVO* MODELS FOR PSORIASIS.

The investigated *in vivo* models in the present thesis are: the tape stripping model (Chapter 2.2), the leukotriene B_4 model (Chapter 3.1) and the relapse of the psoriatic lesion after successful treatment (Chapter 3.2).

ANTIPSORIATIC THERAPY

Knowledge of the cell biological effects of antipsoriatic treatments is essential for the development of new strategies The action spectrum of promising compounds can be differentiated by assessment of their interference with epidermal proliferation, keratinization and inflammation Multiparameter flow cytometry might enable quantitative comparison of different antipsoriatic approaches Furthermore, rational combinations of antipsoriatic therapies might be developed

The third aim of the present thesis is.

(3) TO DEVELOP AND EVALUATE NEW STRATEGIES IN THE TOPICAL TREATMENT OF PSORIASIS

REFERENCES

1 Greaves MW, Weinstein GD Treatment of psoriasis Drug Therapy 1995, 332 581-588

2 van Dooren-Greebe RJ, Lemmens JAM, de Boo T, Hangx NMA et al Prolonged treatment with oral retinoids in adults no influence on the frequency and severity of spinal abnormalities Br J Dermatol 1996, 134 71-76

3 Bahmer F The size of lesions, or point counting as a step towards the solution of the PASI problem Arch Dermatol 1989, 125 1282-1283

4 Marks R, Barton SP, Shuttleworth D, Finlay AY Assessment of disease progress in psoriasis Arch Dermatol 1988, 125 235-240

5 Fredriksson T, Pettersson U Severe psoriasis - oral therapy with a new retinoid Dermatologica 1978, 157 238-244

6 van de Kerkhof PCM On the limitations of the psoriasis area and severity index (PASI) Br J Dermatol 1992, 126 205

7 Ramsay B, Lawrence CM Measurement of involved surface area in patients with psoriasis Br J Dermatol 1991, 124 565-570

8 Strumia R, Altieri E, Romani I, Bettoli V, et al Tacalcitol in psoriasis a video-microscopy study Acta Derm Venereol (Stockh) 1994, 186(Suppl) 85-87

9 Berardesca E, Vignoli GP, Farinelli N, Vignini M, et al Non-invasive evaluation of topical calcipotriol versus clobetasol in the treatment of psoriasis Acta Derm Venereol (Stockh) 1994, 74 302-304

10 Berardesca E, Maibach HI Noninvasive bioengineering assessment of psoriasis Int J Dermatol 1989, 28 157-160

11 Vaillant L, Berson M, Machet L, Callens A, et al Ultrasound imaging of psoriatic skin a noninvasive technique to evaluate treatment of psoriasis Int J Dermatol 1994, 33 786-790

12 Klemp P, Staberg B Cutaneous blood flow in psoriasis J Invest Dermatol 1983, 81 503-506

13 Staberg B, Klemp P. Skin blood flow in psoriasis during Goeckerman or beech tar therapy Acta Derm Venereol (Stockh) 1984, 64: 331-334.
14 Broby-Johansen U, Karlsmark T, Petersen LJ, Serup J. Ranking of the antipsoriatic effect of various topical corticosteroids applied under a hydrocolloid dressing - skin-thickness, blood-flow and colour measurements compared to clinical assessments. Clin Exp Dermatol 1990; 15 343-348.
15 Broby-Johansen U, Kristensen JK. Antipsoriatic effect of local corticosteroids--O2-consumption and blood flow measurements compared to clinical parameters. Clin Exp Dermatol 1989; 14. 137-140.
16 Hull SM, Goodfield M, Wood EJ, Cunliffe WJ. Active and inactive edges of psoriatic plaques: identification by tracing and investigation by laser-Doppler flowmetry and immunocytochemical techniques J Invest Dermatol 1989; 92: 782-785.
17 Goodfield M, Hull SM, Holland DB, Roberts SG, et al. Investigations of the 'active' edge of plaque psoriasis: vascular proliferation precedes changes in epidermal keratin. Br J Dermatol 1994; 131 808-813.
18 Clar E, Cambrai M, Heid E, Grosshans E. Les parametres electrophysiologiques de l'epiderme dans le psoriasis (étude préliminaire). Ann Dermatol Syphiligr 1976, 83: 291-295.
19 Cambrai M, Clar E, Grosshans E, Altermatt C. Skin impedance and phoreographic index in psoriasis. Relationship with action kinetics of three treatments. Arch Dermatol Res 1979, 264 197-211
20 de Jong EMGJ Dynamics of epidermal growth and inflammation in psoriasis. Thesis, Nijmegen, the Netherlands 1993.
21 de Mare S. Proliferation and keratinization in psoriasis Thesis, Nijmegen, the Netherlands 1991.
22 Gerritsen MJP. Dynamics of epidermal growth and keratinization in psoriasis Thesis, Nijmegen, the Netherlands 1995
23 van Erp PEJ, Rijzewijk JJ, Boezeman JBM, Leenders J, et al Flow cytometric analysis of epidermal subpopulations from normal and psoriatic skin using monoclonal antibodies against intermediate filaments. Am J Pathol 1989; 135 865-870.
24 Moldavan A. Photo-electric technique for the counting of microscopial cells Science 1934, 80: 188.
25 Friedman H. The use of ultraviolet light and fluorescent dyes in the detection of uterine cancer by vaginal smear. Am J Obst Gynec 1950; 59: 852.
26 Coons A, Creech H, Jones R. Immunological properties of an antibody containing a fluorescent group. Proc Soc Exptl Biol Med 1941, 47: 200.
27 Dittrich W, Göhde W. Impulsfluorometrie bei Einzelzellen in Suspension. Z Naturforsch 1969; 24: 360-361.
28 Shapiro H. History. In: Practical flow cytometry (Anonymous) 3rd edn., New York Wiley-Liss, Inc. 1995; 43-73.
29 Bonner W, Hulett H, Sweet R, Herzenberg L. Fluorescence activated cell sorting. Rev Sci Instrum 1972; 43: 404-409.
30 Newton J, Camplejohn R, McGibbon D A flow cytometric study of the significance of DNA aneuploidy in cutaneous lesions. Br J Dermatol 1987; 117: 169-174.

31 Randall M, Geisinger R, Kute T, Buss D, et al. DNA content and proliferative index in cutaneous squamous cell carcinoma and keratoacanthoma. Am J Clin Path 1990; 93 259-262.

32 Stenziger W, Suter L, Schumann J. DNA ploidy in congenital melanocyte naevi. suggestive evidence for premalignant changes. J Invest Dermatol 1984; 82. 569-572.

33 Steijlen PM, Hamm H, van Erp PEJ, Johnson JP, Ruiter DJ, Brocker E-B Immunohistologic evidence for the malignant potential of congenital melanocytic nevi. J Invest Dermatol 1989; 92: 366-370.

34 Sondergaard K, Larson JK, Moller U, Christensen IJ, et al. DNA ploidy-characteristics of human malignant melanoma analyzed by flow cytometry and compared with histology and clinical course. Virchows Archiv [B] 1983; 42: 43-52.

35 Kheir S, Bines S, Vonroenn J, Soong S, et al. Prognostic significance of DNA ploidy in stage I cutaneous melanoma. Ann Surg 1988; 207. 455-461.

36 Newton JA, Camplejohn RS, McGibbon DH. The flow cytometry of melanocytic skin lesions. Br J Cancer 1988; 58: 606-609.

37 Kamino H, Ratech H. Improved detection of aneuploidy in malignant melanoma using multiparameter flow cytometry for S100 protein and DNA content. J Invest Dermatol 1989; 93: 392-396.

38 Bauer R, Böhm I. Flowzytometrie in der Dermatologie. Hautarzt 1996; 47· 561-578

39 Le Pecq J, Paoletti C. A fluorescent complex between ethidium bromide and nucleic acids. J Mol Biol 1967; 27: 87-106.

40 Hudson B, Upholt W, Divinny J, Vinogard J. The use of an ethidium bromide analogue in the dye-buyoant density procedure for the isolation of closed circular DNA the variation of the superhelix density of mitochondrial DNA. Proc Natl Acad Sci USA 1969, 62· 813-820.

41 Bauer FW, de Grood RM. Impulse cytophotometry in psoriasis Br J Dermatol 1975; 93 225-227

42 Bauer FW, de Grood RM. Improved technique for epidermal cell cycle analysis Br J Dermatol 1976; 95: 565

43 Larson J, Frentz G, Moller U, Christensen I. A method for flowcytometric cell cycle analysis of normal and psoriatic human epidermis based on a detergent/citric acid technique for suspension of nuclei. Virchows Archiv [B] 1985; 48: 247-259.

44 Staiano Coico L, Gottlieb AB, Barazani L, Carter D. RNA, DNA and cell surface characteristics of lesional and nonlesional psoriatic skin J Invest Dermatol 1987, 88: 646-651.

45 Bauer FW, Crombag NHCMN, de Grood RM, de Jongh GJ. Flow cytometry as a tool for the study of cell kinetics in epidermis. Br J Dermatol 1980, 102 629-639.

46 Bauer FW, Crombag NHCMN, Boezeman JBM, de Grood RM. Flow cytometry as a tool for the study of cell kinetics in skin 2. Cell kinetic data in psoriasis. Br J Dermatol 1981; 104: 271-276.

47 Ramaekers FCS, Puts JJ, Moesker O, Kant A, et al. Antibodies to intermediate filament proteins in the immunohistochemical identification of human tumours· an overview. Histochem J 1983; 15: 691-713

48 Hentzer B, Kobayashi T. Dissociation of human adult epidermal cells by disulfide-reducing agents and subsequent trypsinization. Acta Derm Venereol (Stockh) 1976; 56· 19-25

49 Crombag NHCMN Flowcytometrisch onderzoek van normale en pathologisch veranderde humane epidermis, in het bijzonder van psoriasis Thesis, Nijmegen, the Netherlands 1983
50 McKay IA, Leigh IM Altered keratinocyte growth and differentiation in psoriasis Clinics in Dermatology 1995, 13 105-114
51 Dover R Cell kinetics of keratinocytes In Leigh I, Lane B, Watt F editors The keratinocyte handbook Cambridge Cambridge University Press, 1994 203-234
52 van Erp PEJ, Boezeman JBM, Brons PPT Cell cycle kinetics in normal human skin by in vivo administration of iododeoxyuridine and application of a differentiation marker - implications for cell cycle kinetics in psoriatic skin Anal Cell Pathol 1996, 11 43-54
53 Heenen M, de Martelaer V, Galand P Psoriasis and cell cycle a computer simulation Acta Derm Venereol (Stockh) 1979 Suppl 87 73-76
54 Weiss R, Eichner R, Sun T Monoclonal antibody analysis of keratin expression in epidermal diseases a 48- and 56-kdalton keratin as molecular markers for hyperproliferative keratinocytes J Cell Biol 1984, 98 1397-1406
55 Bauer FW, Boezeman JBM, van Engelen L, de Grood RM, et al Monoclonal antibodies for epidermal population analysis J Invest Dermatol 1986, 87 72-75
56 de Mare S, van Erp PEJ, van de Kerkhof PCM Epidermal hyperproliferation assessed by the monoclonal antibody Ks8 12 on frozen sections J Invest Dermatol 1989 92 130-131
57 de Waal RM, Semeijn JT, Cornelissen MH, Ramaekers FCS Epidermal Langerhans cells contain intermediate-sized filaments of the vimentin type an immunocytologic study J Invest Dermatol 1984, 82 602-604

Chapter 2

TRIPLE-LABELLING FLOW CYTOMETRY AND DEVELOPMENT OF CELL ISOLATION PROCEDURES

2.1 TO-PRO-3 iodide, a novel HeNe laser-excitable DNA stain as an alternative for propidium iodide in multiparameter flow cytometry

Candida A E M van Hooijdonk, Conrad P Glade, Piet E J van Erp,
Department of Dermatology, University Hospital Nijmegen,
The Netherlands
Cytometry 1994; 17:185-189

SUMMARY

A new red emitting fluorophore, TO-PRO-3 iodide (TP3), which is best excited by a HeNe laser (633 nm), has been compared with propidium iodide (PI) for measuring relative DNA content TP3, which has a peak absorbance at 642 nm and emission at 661 nm, has been tested on peripheral blood lymphocytes (PBL) and keratinocytes in a two-laser system As an example, we present a three-color flow cytometric application utilizing TP3 in combination with fluorescein-isothiocyanate (FITC) and phycoerythrin (PE) conjugated to monoclonal antibodies in this paper

A subequilibrium concentration of 1 μM TP3, most preferably used in combination with RNase treatment, showed to be a powerful alternative for DNA amount determination In human- and mouse-Balb/MK-keratinocyte populations with different S-phase fractions, PI and TP3 showed a good correlation Finally, in the triple labelling experiment we clearly demonstrated that TP3 is readily applied to the analysis of binding of two antibodies and relative DNA content simultaneously

INTRODUCTION

For many decades, PI has widely been used as a dye for measuring relative DNA content in single parameter flow cytometry[5 10 11] Since many investigators became interested in multiparameter analysis, PI has also been used in combination with antibody labelling[16] Because of the large spectral overlap of PE and PI fluorescence, simultaneous measurement of FITC, PE and PI has been considered not to be easy[12 13 15] Recent publications showed the use of PI for this purpose [3 4] However, compensation difficulties still remain

In 1984, Zelenin et al. investigated an alternative DNA stain, 7-amino-actinomycin D (7AAD), which has an emission spectrum with a maximum at a longer wavelength compared to PI (> 650 nm)[20]. Although the quantum efficiency is low, it can be used in combination with FITC- and PE-labelled antibodies using only one single (488 nm) excitation beam[11]. The DNA content distributions obtained using 7AAD in this way have been disappointingly broad. So others have chosen to use a dual-beam UV and 488 nm system and have used Hoechst 33342 as the DNA stain[1,14].

Nevertheless, having at our the disposal an inexpensive low-power air-cooled HeNe laser (633 nm), we searched for a new red emitting fluorophore which could be excited with this laser. Recently, a family of new DNA probes with exceptional spectral properties has become available from Molecular Probes, Inc. (Eugene, OR)[7,9]. TP3, which has peak absorbance at 642 nm and emission at 661 nm, is best excited by the HeNe laser at 633 nm. So this makes TP3 a powerful alternative for measuring DNA, and hopefully, it can be used in combination with FITC and PE for three-color flow cytometry. In this paper, we have compared PI and TP3 in a two-laser system and give an example of a triple labelling.

MATERIALS AND METHODS

Cell culture and cell preparation

In this study fixed cell suspensions from lymphocytes, cultured keratinocytes and human skin biopsies were used.

PBL were obtained from a healthy donor and isolated by Ficoll-Isopaque-1077 (Pharmacia, Uppsala, Sweden) density centrifugation. Light density mononuclear cells were washed with PBS, fixed with ice-cold 70% ethanol and stored at -20°C. Human keratinocytes were cultured in keratinocyte growth medium (KGM) and growth arrest was induced either by using keratinocyte basal medium (KBM) or KGM supplemented with transforming growth factor-ß (TGF-ß)[18].

The mouse keratinocyte line, Balb/MK, was kindly provided by Dr. S.A. Aaronson. Balb/MK keratinocytes were either grown exponentially ("cycling" state) or switched to maintenance medium ("quiescent" state) under culture conditions as described earlier[17].

Human skin biopsies, 0.5 cm^2 in size and 0.2 mm in thickness, were obtained from healthy volunteers from which the horny layer was removed by "sellotape stripping"[6].

Biopsies were trypsinized, cell suspensions fixed in ice-cold 70% ethanol and stored at -20°C according to Gommans et al.[8]

Staining

The same staining procedure has been used for PI (Calbiochem, San Diego, CA) as well as for TP3 (Molecular Probes, Inc.). Up to one million fixed cells, either lymphocytes or keratinocytes, were resuspended in 500 µl PBS containing 40 µg/l PI or 1 µM (671 µg/l) TP3 and incubated for 15 min with 50 µl 1% w/v RNase (Sigma Chemicals Co., St. Louis, MO)[2]. In one experiment TP3 concentrations were varied and in another the effect of RNase was tested. Exact conditions are given in the Results and Discussion section. Triple labelling was performed using an IgG1-type monoclonal antibody recognizing the intermediate filament-type protein keratin 10 (RKSE60, provided by Prof. F.C.S. Ramaekers, Dept. of Mol. Cell Biology, University Hospital Maastricht, The Netherlands) and an IgG2a-type monoclonal antibody against another intermediate filament-type protein vimentin (VIM 3B4, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) in combination with TP3. Second-step immunofluorescent staining reagents for mouse IgG1 and IgG2a monoclonal antibodies, conjugated with PE and FITC, respectively, were obtained from Southern Biotechnology Associates, Inc., Birmingham, AL.

Flow cytometry

Samples were analysed on a Coulter Elite flow cytometer (Coulter, Luton, UK). Cells were excited with an air-cooled 488 nm argon laser set at 15 mW (PI-, FITC- and PE staining, forward scatter and right angle scatter signals) and with a 10 mW 633 nm HeNe laser for TP3 staining. The Coulter Elite emission filters were 520-530 nm BP (green; FITC), 555-595 nm BP (orange; PE), 630 nm LP (red; DNA, PI) and 670-680 nm BP (red; DNA, TP3). At least 10,000 cells were analysed per sample. For triple labelling experiments standard electronic compensation was used to remove residual spectral overlap between FITC and PE, and minimal compensation in the case of TP3. Typical settings for three-color fluorescence on the Coulter Elite were 580, 520, 550 V on photomultiplier tubes for green (FL1), orange (FL2), and red (FL3) detectors, respectively; and 2, 21 and 2% compensation for (FL1-%FL2), (FL2-%FL1), (FL3-%FL2), respectively.

The ratio area/peak discriminates between artifacts due to doublets of diploid cells and real single tetraploid (or late S-phase) cells[2]. Multicycle™ software (Phoenix Flow

Systems, San Diego, CA) was used to calculate the percentage S-phase positive cells from DNA histograms of PI as well as of TP3.

RESULTS AND DISCUSSION

TP3 showed to be an excellent alternative for measurement of relative DNA content. In addition to its similar staining characteristics to PI, it was superior as DNA stain in multiparameter flow cytometry when combined with the use of two antibodies (FITC- and PE-conjugated). The need for a second laser is only a minor disadvantage, since there are many excellent research flow cytometers which are equipped as standard with a low-power HeNe laser, for instance the Coulter Elite flow cytometer[19]. 7AAD and Hoechst 33342 have been proposed as alternatives for PI in multiparameter flow cytometry[1,11,14,20]. However, in our hands, using epithelial cells, 7AAD gave unacceptable high coefficients of variation (CVs) (data not shown). The other alternative, Hoechst 33342 staining, requires the use of an expensive UV laser, which is not a standard component in many commercial flow cytometers.

PBL were used to test different concentrations of TP3 in a range from 10 nM to 10 µM. A linear increase in peak position of the TP3 signal up to 2 µM was shown. Above this concentration, the peak positions decreased, probably because the blue color of the dye interferes with the measurement. So therefore we decided to use a subequilibrium concentration of 1 µM TP3 in our experiments.

In Figure 1 the effect of RNase treatment on the DNA distributions is shown. Figure 1B. demonstrates that lower CVs could clearly be obtained by adding 1% w/v RNase to the TP3-containing cell suspensions. Experimental conditions were identical to those when using PI[2].

Furthermore, we have used human and mouse keratinocytes, for comparison of PI and TP3 with respect to S-phase determination. Human keratinocytes were grown in KGM. Additionally, these cells were growth arrested in KGM with TGF-ß or KBM, and keratinocytes were restimulated with KGM after growth arrest in KGM with TGF-ß. Mouse-Balb/MK-keratinocytes were either grown exponentially or switched to a maintenance medium. The aim of the different culture conditions was to obtain cell populations with different S-phase fractions. Figure 2 demonstrates the direct comparison of histograms using PI and TP3. It clearly shows a very good correlation between the percentage S-phase positive cells using either PI or TP3 under all our

experimental conditions. This can easily be explained by the similarity of the two fluorochromes, both intercalating in ds DNA[9].

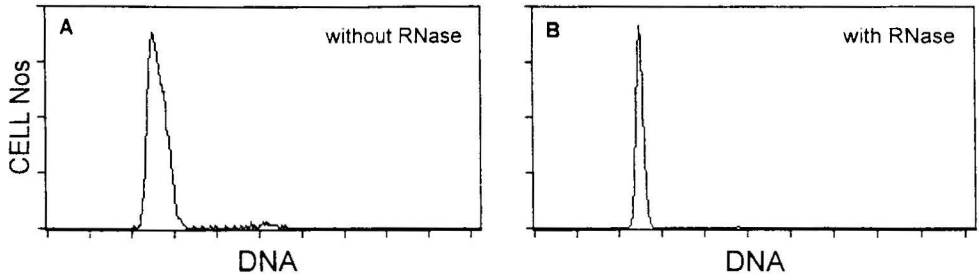


Figure 1. The effect of 1%w/v RNase added to TP3-containing cell suspensions of PBL. Two histograms are shown: (**A**) without (CV = 7.9%) and (**B**) with RNase treatment (CV = 3.6%).

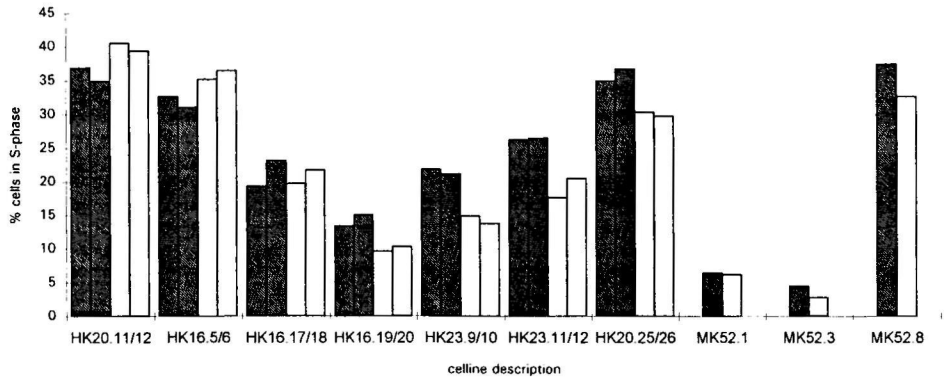


Figure 2. Comparison between TP3 (dark-shaded bars) and PI (light-shaded bars) with respect to cells in S-phase of the cell cycle. Human- (HK) and mouse-Balb/MK-keratinocytes (MK) were cultured under different experimental conditions, resulting in different S-phase fractions.

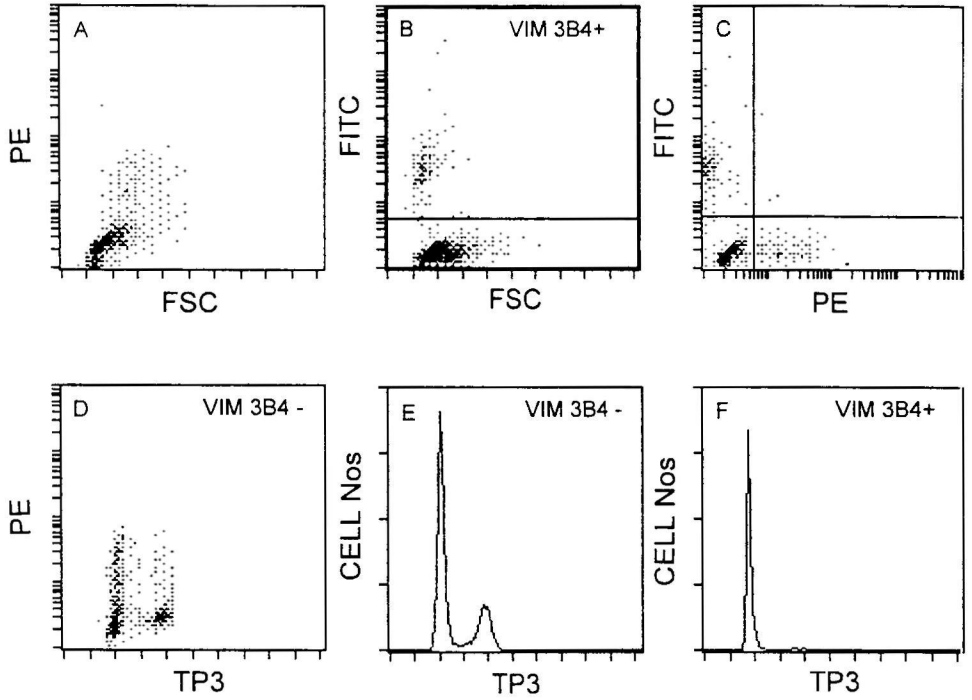


Figure 3. An example of a three-color flow cytometric analysis. Epidermal cell suspensions were labelled with two monoclonal antibodies and TP3. **A:** FSC (forward scatter) vs. PE (RKSE60). **B:** FSC (forward scatter) vs. FITC (VIM 3B4). **C:** PE (RKSE60) vs. FITC (VIM 3B4). **D:** TP3 (DNA) vs. PE (RKSE60) of the VIM 3B4-negative cell population. **E:** TP3 (DNA) histogram of the VIM 3B4-negative cell population. **F:** TP3 (DNA) histogram of the VIM 3B4-positive cell population (non-keratinocytes).

An example of a triple labelling is demonstrated in Figure 3 . Two antibodies, recognizing different intermediate filament-type proteins, were used to separate an epidermal cell suspension into three different subpopulations. Differentiated keratinocytes were keratin 10 (RKSE60, PE)-positive and all non-keratinocytes were vimentin (VIM 3B4, FITC)-positive. Relative DNA content was measured using 1μM TP3. Typical CVs for the diploid TP3 peak ranged from 3 to 4% in vimentin-positive cells and 4 to 8% in keratinocytes. These results were similar to results obtained with PI in double labelling experiments[16]. The advantage of using TP3 incombination with FITC and PE is that there is no need for fluorescence compensation for the TP3 signal. The DNA signal is plotted on a linear scale and interference of PE and/or FITC

fluorescence would result in unreliable DNA histograms. For this reason we think the combination of FITC-PI-Cy5 is less useful.

In summary, we believe that the HeNe laser-excitable fluorochrome TP3 is an excellent alternative for PI in measuring relative DNA content. Since electronic compensation of the DNA signal is not really necessary, analysis of the binding of two antibodies and a reliable DNA measurement can be performed simultaneously.

REFERENCES

1 Arndt-Jovin DJ, Jovin TM Analysis and sorting of living cells according to deoxyribonucleic acid content J Histochem Cytochem 1977, 25 585-589

2 Bauer FW, Boezeman JBM Flow cytometric methods in human skin with respect to cell cycle kinetics In Psoriasis cell proliferation, Wright NA and Camplejohn RS (eds) Churchill Livingstone, Edinburgh, 1983, pp 104-116

3 Begg AC, Hofland I Cell kinetic analysis of mixed populations using three-color fluorescence flow cytometry Cytometry 1991, 12 445-454

4 Corver WE, Cornelisse CJ, Fleuren GJ Simultaneous measurement of two cellular antigens and DNA using fluorescein-isothiocyanate, R-phycoerythrin, and propidium iodide on a standard FACScan Cytometry 1994, 15 117-128

5 Crissman HA, Steinkamp JA Rapid simultaneous measurement of DNA, protein and cell volume in single cells from large mammalian cell populations J Cell Biol 1973, 59 766-771

6 de Mare S, van Erp PE, Ramaekers FC, van de Kerkhof PC Flow cytometric quantification of human epidermal cells expressing keratin 16 in vivo after standardized trauma Arch Dermatol Res 1990, 282 126-130

7 Doornbos RMP, de Grooth BG, Kraan YM, van der Poel CJ, Greve J Visible diode lasers can be used for flow cytometric immunofluorescence and DNA analysis Cytometry 1994, 15 267-271

8 Gommans JM, Bergers M, van Erp PEJ, van den Hurk JJMA, Mier PD, Roelfzema H Studies on the plasma membrane of normal and psoriatic keratinocytes I Preparation of material and morphological characterization Br J Dermatol 1979, 101 407-412

9 Hirons GT, Fawcett JJ, Crissman HA TOTO and YOYO New very bright fluorochromes for DNA content analyses by flow cytometry Cytometry 1994, 15 129-140

10 Krishan A Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining J Cell Biol 66 1975, 188-193

11 Rabinovitch PS, Torres RM, Engel D Simultaneous cell cycle analysis and two-color surface immunofluorescence using 7-amino-actinomycin D and single laser excitation applications to study of cell activation and the cell cycle of murine Ly-1 B cells J Immunol 1986, 136 2769-2777

12 Schmid I, Krall WJ, Uittenbogaart CH, Braun J, Giorgi JV Dead cell discrimination with 7-amino-actinomycin D in combination with dual color immunofluorescence in single laser flow cytometry Cytometry 1992, 13 204-208

13 Schmid I, Uittenbogaart CH, Giorgi JV A gentle fixation and permeabilization method for combined cell surface and intracellular staining with improved precision in DNA quantification Cytometry 1991, 12 279-285

14 Shapiro HM Parameters and probes In Practical Flow Cytometry, Second Edition, Alan R Liss, Inc , New York, 1988, pp 133-137

15 Toba K, Winston EF, Bray RA Improved staining method for the simultaneous flow cytofluorometric analysis of DNA content, S-phase fraction, and surface phenotype using single laser instrumentation Cytometry 1992, 13 60-67

16 van Erp PEJ, Rijzewijk JJ, Boezeman JBM, Leenders J, de Mare S, Schalkwijk J, van de Kerkhof PCM, Ramaekers FCS, Bauer FW Flow cytometric analysis of epidermal subpopulations from normal and psoriatic skin using monoclonal antibodies against intermediate filaments Am J Pathol 1989, 135 865-870

17 van Hooijdonk CAEM, van Erp PEJ, Freund RL, de Jongh GJ, Schalkwijk J, van de Kerkhof PCM, Mier PD Cell kinetic characterization of the EGF-dependent Balb/MK line using flow cytometric analysis of DNA content and iododeoxyuridine incorporation Cell Prolif 1993, 26 567-576

18 van Ruissen F, van Erp PEJ, de Jongh GJ, Boezeman JBM, van de Kerkhof PCM, Schalkwijk J Cell kinetic characterization of growth arrest in cultured human keratinocytes J Cell Sci 1994, 107 2219-2228

19 Waggoner AS, Ernst LA Fluorescent reagents for flow cytometry In Clinical Flow Cytometry Principles and Applications, Bauer KD, Duque RE, and Shankey TV (eds) Williams & Wilkins, Baltimore, 1993, pp 111-116

20 Zelenin AV, Poletaev AI, Stepanova NG, Barsky VE, Kolesnikov VA, Nikitin SM Zhuze AL, Gnutchev NV 7-Amino-actinomycin D as a specific fluorophore for DNA content analysis by laser flow cytometry Cytometry 1984, 5 348-354

2.2 Characterization of epidermal cell suspensions prepared from normal and hyperproliferative human skin using an optimized thermolysin-trypsin protocol

Conrad P Glade, Bianca A M P A Seegers, Etienne F J Meulen, Candida A E M van Hooijdonk, Piet E J van Erp, Peter C M van de Kerkhof,
Department of Dermatology, University Hospital Nijmegen, The Netherlands
Arch Dermatol Res 1996; 288; 203-210

SUMMARY

Reliable flow cytometric analysis of normal and diseased skin requires pure epidermal single-cell suspensions Several methods to separate the dermis from the epidermis are available The proteolytic enzyme thermolysin separates the epidermis from the dermis at the lamina lucida and therefore permits reliable dermoepidermal separation In the present study an optimized cell isolation procedure using thermolysin and trypsin is described, which is particularly suitable for punch biopsies A 16–20-h (overnight) incubation of biopsies taken from normal and hyperproliferative skin with thermolysin (0 5 mg/ml) at 4°C produced a selective separation of the dermis and epidermis After a 30-min trypsin incubation (0 25 mg/ml) at 37°C a cell suspension was prepared which was characterized by minimal cell damage (cellular debris and clumps), a high recovery of basal cells and high quality DNA histograms Furthermore, dermal contamination was very low The thermolysin–trypsin separation methodology followed by triple-labelling flow cytometry provided a precise quantification of the percentage of keratin 10-positive cells, vimentin-positive cells and cells in S- and G_2M phase Proliferative activity was measured selectively in the basal, the suprabasal and the non-keratinocyte compartment at various time intervals during epidermal regeneration after sellotape stripping In contrast to the non-keratinocytes, the percentage of cells in S- and G_2M phase in the basal keratinocytes and in the suprabasal compartment increased 44–48 h after stripping The increased proliferation following tape stripping was paralleled by an increased invasion of vimentin-positive cells into the epidermis and preceded by a decreased number of keratin 10-positive cells Thermolysin–trypsin separation followed by three-colour flow cytometry permits a highly selective characterization of normal and hyperproliferative epidermis

INTRODUCTION

Flow cytometry is a valuable approach for quantifying the behaviour of normal and diseased epidermis under various conditions[2 5 9] A prerequisite for accurate and reliable quantification is a high quality of epidermal single-cell suspensions In this respect it is essential that the biopsy specimens obtained for preparation of these suspensions always contain the whole epidermis Therefore, in hyperproliferative skin diseases such as psoriasis, dermatotome (0 4 mm depth) or preferably punch biopsies are required However, contamination with dermal cells is inevitable if dermoepidermal separation is not part of the cell isolation procedure

In the past, dermoepidermal separation has been achieved using different methods such as suction blisters [16] (mechanical), sodium chloride[30] (chemical) and proteolytic enzymes, such as trypsin[13], dispase[17] and thermolysin[29] Enzymological methods are preferred as they are site–specific, and less time consuming Until recently we have used incubation with trypsin in combination with dithioerythritol (DTE) to obtain dermoepidermal separation and dissociation of the keratinocytes[12 14] A limitation of this method is the fact that the level of separation is dependent on factors such as thickness of the biopsy, purity of the preparation, incubation time and temperature[10 25] Dispase is often to obtain samples free from dermal contaminants[2 17 18], however, changes in the basal compartment and impaired viability have been described[23 24] Thermolysin appears to produce a selective separation of the dermoepidermal junction, providing an epidermal sheet with a complete basal layer[29] Subsequent incubation with trypsin provides single-cell suspensions suitable for flow cytometric analysis[10] Various incubation times and temperatures have been proposed by different authors[10 25 29 30] So far, thermolysin–trypsin separation has not been validated in hyperproliferative skin

Multiparameter flow cytometry enables simultaneous measurement of different cell parameters[9] Until now three-colour flow cytometry combining dual immunophenotyping with quantitative DNA analysis has been impeded by a considerable spectral overlap of the fluorochromes used Recently we have demonstrated that this problem can be overcome by using the new DNA dye TO-PRO-3 iodide (TP3) in combination with the fluorochromes phycoerythrin (PE) and fluorescein isothiocyanate (FITC)[15] In the present study the contamination with dermal cells and the relative numbers of basal cells after dermoepidermal separation was quantified using double-labelling flow cytometry In epidermal cell suspensions prepared from normal and hyperproliferative skin, a triple-labelling procedure was

applied with simultaneous quantification of the basal and differentiated keratinocytes, cells of mesenchymal origin and the DNA content of these epidermal subpopulations.

The aim of the present study was to validate a combined thermolysin–trypsin method on normal skin and hyperproliferative skin. Psoriatic lesions and recovery from tape stripping were studied using flow cytometry as conditions characterized by epidermal hyperproliferation. In particular the following issues were addressed: (1) optimization of the thermolysin–trypsin separation procedure in normal and psoriatic skin; (2) quantification of epidermal cell characteristics in biopsies taken from normal skin following both separation methods using triple-labelling flow cytometry; and (3) analysis of epidermal hyperproliferation induced by tape stripping using triple-labelling flow cytometry following thermolysin–trypsin separation.

MATERIALS AND METHODS

Subjects

Skin samples were obtained from 26 healthy volunteers (16 males, 10 females, age range 18–29 years) without signs or history of skin diseases and from the lesional skin of 6 psoriasis vulgaris patients (3 males, 3 females, age range 30–64 years). All subjects had given informed consent prior to biopsy.

Biopsy and tape stripping procedure

Dermatotome biopsies were taken from normal and lesional psoriatic skin[12]. After induction of local anaesthesia with ethyl chloride spray (Medica, 's Hertogenbosch, The Netherlands), a small dermatotome (Coriotome 6B333, Aesculap, Tuttlingen, Germany) with a metal guard was used to obtain skin samples with an area of 1 cm^2 and a thickness of 0.2 mm (normal skin; n=10) or 0.4 mm (psoriatic skin; n=6). Punch biopsies (3 mm) were taken from normal skin (n=7), from lesional psoriatic skin (n=6) and in the tape stripping experiment (n=28). Local anaesthesia was induced with Xylocaine/adrenaline 1:100 000.

Tape stripping was carried out on the back of seven healthy volunteers[8]. By repeated applications of Sellotape adhesive tape the stratum corneum was removed from four test areas (± 2 cm^2) until the surface was glistening. In total, five punch biopsies were obtained from each volunteer, before tape stripping and after 8, 24, 44 and 48 h. At each visit erythema, induration and desquamation were assessed clinically using a five-point scale (0 = not present, 1 = slight, 2 = moderate, 3 = severe and 4 = very severe).

Cell isolation procedure

The trypsin cell isolation procedure has been described previously[12]. In brief, after a one-step incubation of the skin specimen in phosphate-buffered saline (PBS) containing 0.25 mg/ml trypsin (Sigma T-8253, St. Louis, Mo., USA) and 3.0 mg/ml DTE (Sigma, D-8255) for 30 min at 37°C, the dermis was separated with forceps in PBS containing 10% heat-inactivated newborn calf serum (HINCS, Life Technologies, Paisley, UK). The remaining epidermis was then gently mixed on a vortex to separate the keratinocytes, resulting in a single-cell suspension. The stratum corneum was discarded. A limitation of this method is that it is not applicable to punch biopsy specimens.

The two-step thermolysin–trypsin separation procedure was tested under different concentrations, incubation times and temperatures for both thermolysin (Sigma P-1512) and trypsin as described in the Results section. After thermolysin incubation (0.5 mg/ml dissolved in PBS with Ca^{2+} and Mg^{2+}; Seromed, Berlin, Germany), the dermis and epidermis were separated using fine forceps. This separation was visualized in normal and psoriatic skin by routine HE staining. Subsequent trypsin–DTE incubation provided single-cell suspensions.

Cells were fixed in 1–3 ml ice-cold ethanol (70% v/v) and kept at –20°C. To be able to compare the percentage of intact cells (without debris and clumps) the suspensions were not filtered before staining and flow cytometric analysis.

Flow cytometric analysis

Flow cytometric validation of the separation method consisted of double-labelling procedures. To obtain information on contamination with dermal cells, propidium iodide (PI; Calbiochem, San Diego, Calif., USA) staining was combined with staining with a monoclonal antibody against the intermediate filament vimentin (Vim3B4, Novocastra Laboratories, Newcastle upon Tyne, UK; see Table 1). The relative amount of basal and suprabasal cells present in the suspensions was quantified by simultaneous staining with the monoclonal mouse antibodies RCK102 and RKSE60 (gifts from Prof. F.C.S. Ramaekers, Department of Molecular Biology, University of Maastricht, The Netherlands) as primary antibodies. Rabbit antimouse IgG conjugated with fluorescein isothiocyanate (RAM-FITC, Dakopatts, Copenhagen, Denmark) was used as secondary antibody in these experiments. Cell suspensions were prepared from dermatotome and punch biopsies from normal and psoriatic skin with different

separation conditions. From each suspension two samples containing about 1×10^5 ethanol-fixed cells were washed in PBS, centrifuged (5 min, 3000 rpm) and resuspended in 500 µl PBS containing either Vim3B4 (1 50 dilution) or RCK102 (1 30 dilution) and RKSE60 (1.15 dilution) After 30 min incubation in the dark at room temperature the cells were washed in PBS containing 1% HINCS, centrifuged and resuspended in 500 µl PBS containing RAM-FITC and HINCS (both 1.50 dilution). After 15 min incubation at 5°C the cells were washed and centrifuged as before and finally resuspended in 500 µl PBS containing 40 µg/ml PI and 0.1% RNase (Sigma, R-4875) and kept for 10 min in the dark..

Table 1. Primary antibodies used in flow cytometric analysis

Primary antibody	Directed against	Present in	Dilution	Source	References
Vim3B4	Vimentin	Mesenchymal cells	1 50	Novocastra	20
RCK102	Keratins 5 and 8	Basal keratinocytes	1 30	Pathology Department	28
RKSE60	Keratin 10	Suprabasal keratinocytes	1 15	Pathology Department	9

A triple-labelling procedure was used to quantify epidermal cell characteristics in normal and hyperproliferative skin. In preliminary experiments specificity and optimal concentrations of the antibodies were tested We have described the staining procedure previously[12] The DNA content was measured using the new DNA fluorochrome TP3 (Molecular Probes, Eugene, Ore., USA). TP3 intercalates with double-stranded DNA and permits the measurement of the proliferative activity of cells by quantification of the percentage of cells in S- and G_2M phase. As TP3 also binds to RNA to some extent, it was used in combination with RNase. Primary intermediate filament antibodies were RKSE60 (mouse, IgG_1) and Vim3B4 (mouse, IgG_{2a}). The second step of the indirect immunofluorescent staining was performed with monoclonal goat antibodies against mouse IgG_1 and IgG_{2a}, conjugated with PE and FITC, respectively (Southern Biotechnology Associates, Birmingham, Ala , USA) Usually suspensions containing about $1-2 \times 10^5$ cells were used for flow cytometric analysis

From each sample 5000 gated cells were measured and analysed using an EPICS® Elite flow cytometer (Coulter, Luton, UK) equipped with a dual laser system PE,

FITC and PI were excited with an air-cooled argon ion laser (15 mW, 488 nm). TP3 was excited with a HeNe laser (10 mW, 633 nm). Fluorescence was measured using bandpass filters of 520–530 nm (green, FITC), 555–595 nm (orange, PE), and 670–680 nm (red, TP3) and a longpass filter of 630 nm (orange–red, PI). The area/peak ratio of the red signal (DNA) was used to discriminate between doublets of diploid cells and real single tetraploid cells[4]. After setting appropriate gates with the EPICS® Elite software, the percentages of cells expressing vimentin, and keratins 5, 8, and 10 were calculated. Using Multicycle™ software (Phoenix Flow Systems, San Diego, Calif., USA) the percentages of basal and suprabasal keratinocytes and of non-keratinocytes in S- and G_2M phase of the cell cycle were calculated from DNA histograms.

RESULTS

Dermoepidermal separation and cell isolation

Experiments were carried out to establish the optimal analytical procedures, to achieve a selective separation of the dermis and epidermis and a maximum number of intact epidermal cells. After thermolysin incubation for 1–2 h at 4°C of punch and dermatotome biopsies taken from both normal and psoriatic skin no reliable dermoepidermal separation was possible (fluorescence microscopic control). A 2-h thermolysin incubation at 37°C did not provide sufficient separation in a punch biopsy from psoriatic skin. In contrast, the following conditions proved to result in optimal cell suspensions: an overnight (16–20-h) incubation with thermolysin (0.5 mg/ml) at 4°C, followed by a 30-min trypsin incubation (0.25 mg/ml) at 37°C. Figure 1 illustrates the typical histological appearance of dermoepidermal separation in punch biopsies from normal skin and from lesional psoriatic skin. It can be seen that the separation selectively involved the dermoepidermal junction without dissociation of the individual keratinocytes. The complete basal cell layer was present in the epidermal compartment without any dermal contamination.

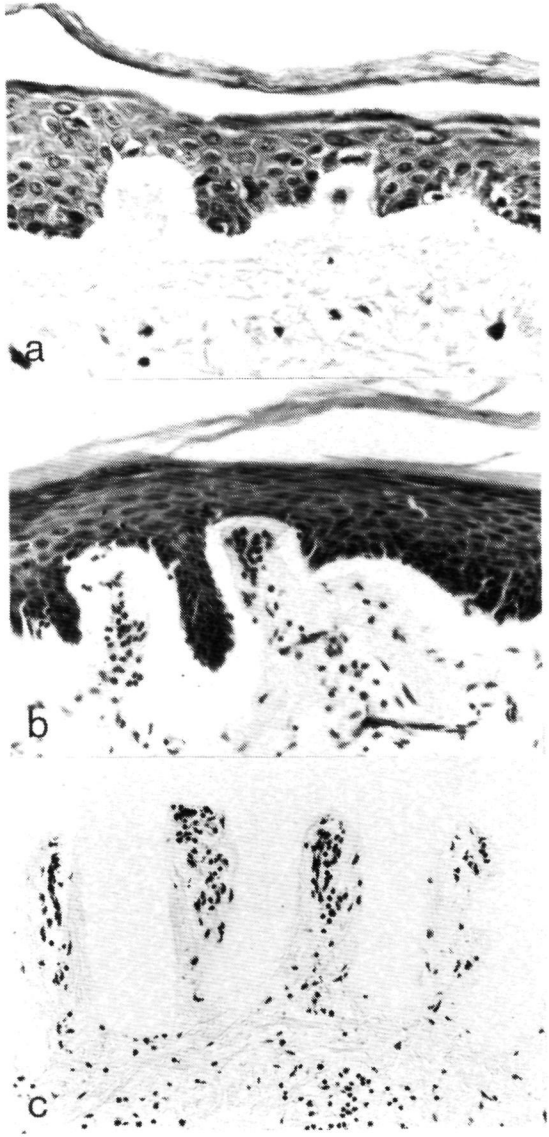


Figure 1a-c. Dermoepidermal separation obtained after incubation of punch biopsies from **(a)** normal skin and **(b)** psoriatic skin with thermolysin (0.5 mg/ml) overnight (16 h) at 4°C). **c** After removal of the psoriatic epidermis from a thermolysin-treated biopsy the remaining dermis is complete (**a** ×400, **b** × 200)

Table 2 summarizes the flow cytometric analysis (double-labelling) of the epidermal sheets prepared from normal and lesional psoriatic skin using trypsin versus thermolysin–trypsin separation methods. It can be seen that the numbers of vimentin-positive cells and the number of naked nuclei was substantially elevated in the preparation of psoriatic skin using the trypsin separation method. After 2 h thermolysin incubation at 4°C (dermatotome biopsy, normal skin) a significant loss of keratin 5-positive keratinocytes, i.e. basal cells, was found. However, using the optimized thermolysin–trypsin method the number of keratin 5-positive keratinocytes tended to be greater than with the trypsin method.

Table 2. Flow cytometric analysis of epidermal cell suspensions prepared from normal and lesional psoriatic skin using trypsin (*t*) and thermolysin–trypsin (*tt*) separation methods.

Biopsy procedure	Skin	Separation method	Vimentin-positive cells (%)	Keratin 10-positive keratinocytes (%)	Keratin 5-positive keratinocytes (%)	Naked nuclei (%)[a]
Dermatotome	Normal	t	5.9	65.6	28.0	0.5
Dermatotome	Psoriasis	t	9.1	26.3	54.7	9.9
Dermatotome	Normal	tt (2 h)	5.9	90.5	3.0	0.6
Punch	Normal	tt (16 h)	7.1	48.1	38.4	6.4
Punch	Psoriasis	tt (16 h)	6.3	29.2	60.8	3.7

[a] % naked nuclei = 100% - (% keratin 5-positive cells + % keratin 10-positive cells + % vimentin-positive cells)

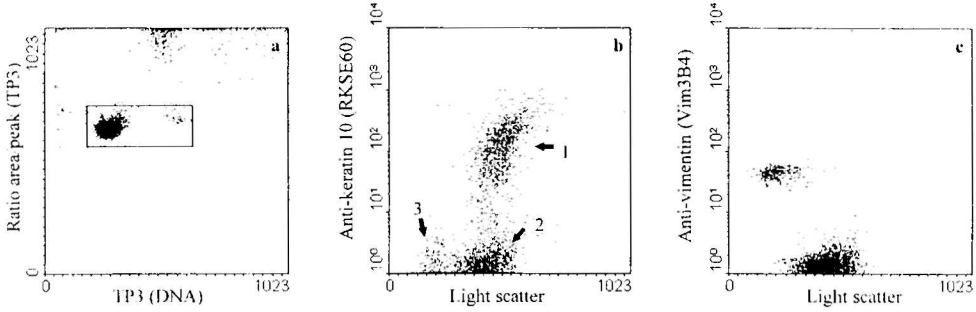


Figure 2a-c. Discrimination of different epidermal subpopulations with triple-labelling flow cytometry. **a** TP3 (DNA content, red signal) versus the area/peak ratio of the red signal; **b** Light scatter versus log orange fluorescence (keratin 10; for explanation of subpopulations 1, 2 and 3, see text); **c** Light scatter versus log green fluorescence (vimentin).

Quantification of the epidermal cell characteristics of normal skin

Representative flow cytometric dot plots obtained by the triple-labelling procedure are given in Fig. 2. Inside the gate (Fig. 2a) all cells with diploid to tetraploid DNA content are present. Cell debris (less DNA content) and clumps (higher area/peak ratio of the TP3 signal) are excluded from the analysis. Antikeratin 10 staining (RKSE60, Fig.2b) resulted in discrimination of keratin 10-positive cells (1), i.e. suprabasal keratinocytes, from keratin 10-negative cells (2), i.e. basal keratinocytes Fig. 2b also shows a subpopulation of keratin 10-negative cells with a smaller size (light scatter) (3). However, no clear separation of this subpopulation was achieved. With respect to antivimentin staining, (Vim3B4) two distinct epidermal subpopulations were discriminated: a vimentin-positive population with small cell size and a vimentin-negative population with larger cell size (Fig. 2c). The first population corresponds to the cells from subpopulation 3 in Fig. 2b. The second population represents all keratinocytes. Cell cycle analysis was performed on histograms representing the germinative layer of the epidermis after exclusion of vimentin- and keratin 10-positive cells by software gating. The flow cytometric quantification of the different epidermal subpopulations using the trypsin separation protocol on dermatotome biopsies versus the thermolysin–trypsin protocol on punch biopsies is summarized in Table 3. It can be seen that the population separated with thermolysin–trypsin was characterized by a decreased percentage of basal cells in S and G_2M phases ($P = 0.012$, *t*-test assuming equal variances, two-tailed), an increased percentage of intact cells ($P < 0.01$), a decreased coefficient of variation of the G_1 peaks ($P = 0.016$), and a slightly decreased percentage of vimentin-positive ($P = 0.79$) and keratin 10-positive cells ($P = 0.56$).

Table 3. Flow cytometric comparison of the trypsin (*t*) and the thermolysin-trypsin (*tt*) cell isolation procedure on normal skin (mean ± SEM)

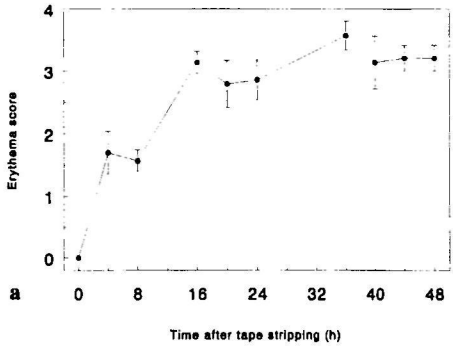
Biopsy procedure	Separation method	Basal cells in $S+G_2M$ phase (%)	Intact cells (%)[a]	Coefficient of variation (G_1-peak)	Vimentin-positive cells (%)	Keratin 10-positive keratinocytes (%)
dermatotome (n=10)	t	8.8 ± 0.8	69 ± 2.8	8.0 ± 0.3	7.5 ± 1.1	61.5 ± 4.5
punch (n=7)	tt	5.5 ± 0.9	88 ± 2.6	6.6 ± 0.5	7.2 ± 0.6	57.8 ± 3.4

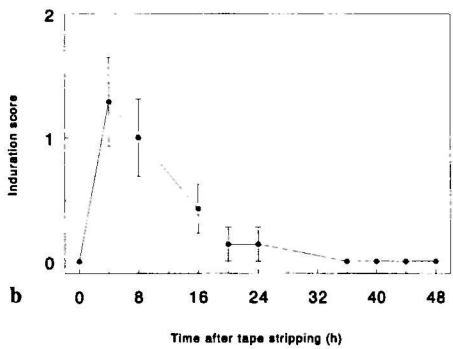
[a] % intact cells = % particles with DNA content (2c-4c) without clumps or debris

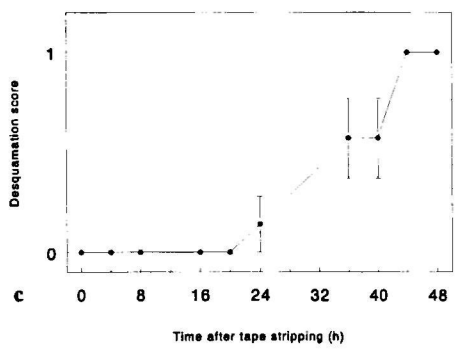
Quantification of the epidermal cell characteristics of normal skin following tape stripping

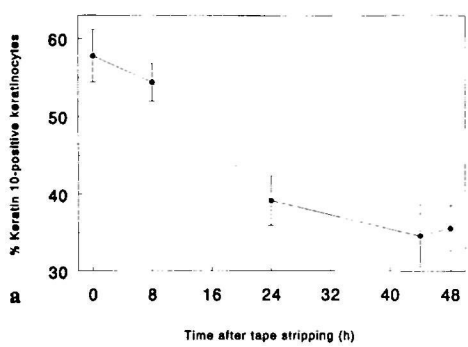
In preliminary experiments flow cytometric analysis was performed on cell suspensions prepared from dermatotome biopsies taken at different intervals after tape stripping using the standard trypsin separation method. Following trypsinization, a reliable separation of dermis from epidermis turned out to be virtually impossible in these thin specimens with a partially to totally removed stratum corneum and hardly any dermis present. This was confirmed by the flow cytometric analysis which gave very inconsistent results especially with respect to the numbers of vimentin- and keratin 10-positive cells (unpublished data). In contrast, following incubation of punch biopsies taken at different intervals after tape stripping with thermolysin–trypsin the dermoepidermal separation was reliable, and pure epidermal single-cell suspensions could be prepared.

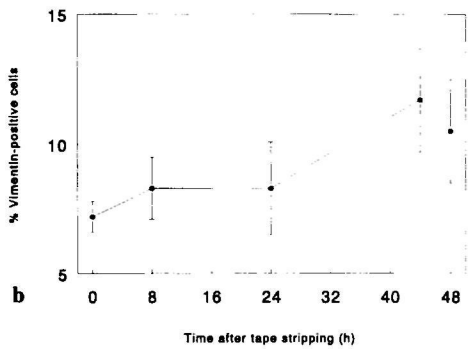
None of the seven healthy volunteers complained as a result of the tape stripping study. The clinical scores at different time intervals after tape stripping are shown in Fig. 3. Maximum erythema was observed 36 h after tape stripping. The triple-labelling flow cytometric analysis of the dynamics of the tape stripping model is summarized in Fig. 4. A significant decrease in the relative number of keratin 10-positive keratinocytes was observed at 24, 44 and 48 h after tape stripping ($P < 0.01$, paired *t*-test, two-tailed; Fig. 4a). The relative number of vimentin-positive cells increased after tape stripping with a maximum value at 44 h ($P < 0.08$; Fig. 4b). With respect to epidermal proliferation the number of basal cells in S and G_2M phases increased significantly at 44 and 48 h after tape stripping ($P < 0.01$). Four volunteers showed a peak value after 44 h. The peak value (all volunteers) was 46.7 ± 2.5 (mean ± SEM). Remarkably, a less pronounced but still substantial increase in proliferative activity was also observed in the suprabasal compartment of the epidermis ($P < 0.01$, at 44 and 48 h). The proliferative activity of the non-keratinocytes in the epidermis was not influenced by tape stripping (Fig. 4c).











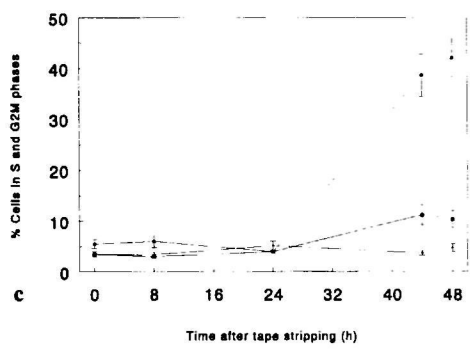


Figure 3a-c. Clinical scores after Sellotape stripping (mean ± SEM; 0 = not present, 1 = slight, 2 = moderate, 3 = severe and 4 = very severe). **a** Erythema; **b** induration; **c** desquamation

Figure 4a-c. Flow cytometric analysis of the dynamics of the tape stripping model (mean ± SEM). **a** Percentage of keratin 10-positive keratinocytes; **b** percentage of vimentin-positive cells; **c** percentage of basal (●) and suprabasal (■) keratinocytes and of vimentin-positive cells (◆) in S and G_2M phases.

DISCUSSION

The epidermis and dermis of biopsies taken from normal and hyperproliferative skin can be separated completely following a combined thermolysin–trypsin digestion method. In the present study the optimal conditions for this method were developed. Furthermore, a reproducible and quantitative analytical method was described to measure simultaneously inflammation, differentiation and proliferation in epidermal cell suspensions.

The separation of the dermis from the epidermis has a major influence on the composition of the epidermal cell suspension, in particular in the inflamed skin of psoriasis. Thermolysin is a proteolytic enzyme which cleaves the lamina lucida and is therefore, from a theoretical point of view, an interesting tool to separate the dermis from the epidermis. In contrast to the findings of Walzer et al.[29], our results indicate that a 1–2-h incubation with thermolysin at 4°C does not provide a reliable dermoepidermal separation. This is supported by Willsteed et al.[30] who observed intraepidermal separation in four of five cases after thermolysin incubation for 1 h at 4°C. Even incubation for 2 h at 37°C, as used by Germain et al.[10] does not provide a reliable separation in psoriatic skin. The optimized procedure for punch biopsies included an overnight (16–20-h) incubation with thermolysin at 4°C[24]. Figure 1 shows that a selective dermoepidermal cleavage can be achieved in punch biopsies following the optimized thermolysin procedure. In psoriatic skin, in particular, this method proved to reduce dermal contamination, to preserve the integrity of epidermal cells and to increase the relative number of basal cells. An intriguing observation was the decrease in the percentage of vimentin-positive cells in the biopsies from psoriatic skin. The inflammatory infiltrate cells in psoriatic lesions contribute to an absolute increase in the vimentin-positive cells. However, in psoriatic plaques the number of germinative cells per surface area has been estimated to be increased by a factor of 7 and the number of suprabasal cells by a factor of 3[3]. This implies that an absolute increase in mesenchymal cells may not represent a relative increase in psoriatic plaques, due to the pronounced increase in keratinocyte populations.

In multiparameter flow cytometry, the DNA fluorochrome TP3 has the advantage of minimal spectral overlap with PE. This is in contrast to other DNA dyes, such as 7-amino-actinomycin D or PI, with which measurements are substantially influenced by the electronic compensation for this overlap[15]. With the three-colour flow cytometric approach using TP3, high quality DNA histograms were obtained. Furthermore, discrimination of different epidermal subpopulations allowed a highly selective

quantification of the DNA content in basal, suprabasal and mesenchymal cells. Using the trypsin method and the combined thermolysin–trypsin method the subpopulations in epidermal cell suspensions prepared from normal skin were analysed. The percentages of vimentin-positive cells and keratin 10-positive keratinocytes (Table 3) are in accordance with previous results[9,19].

Significantly fewer basal cells with hyperdiploid DNA content were present in cell suspensions from normal skin prepared according to the thermolysin procedure (5.5%) compared with the trypsin procedure (8.8%). This implies that after thermolysin–trypsin separation, despite the presence of more basal cells, a lower proliferative activity was measured in the germinative compartment. One explanation for this observation might be the presence of a larger quantity of clumps in the cell suspensions prepared with only trypsin, mimicking cells with a more than diploid DNA content. Indeed, the mean percentage of intact cells was 88% in the cell suspensions isolated according to the thermolysin–trypsin protocol, whereas this percentage was only 69% after trypsin separation. Another explanation might be the lack of proliferative activity of exclusively those basal cells that were preserved with the thermolysin protocol. As we cannot exclude the possibility that the germinative layer contains subpopulations with different proliferating potential, there might exist nonproliferating or very slowly proliferating epidermal cells in the basal layer which were lost in the trypsin separation procedure. These cells might correspond to the stem cells described by Potten and Morris[22]. Future research should resolve this question.

In previous flow cytometric studies the percentage of cells in S and G_2M phases in suspensions prepared from normal skin varied from 2.9% to 7.0%[5,6,9,19]. However, in those studies dermoepidermal separation was obtained with trypsin only, which might have resulted in a relatively high number of clumps and therefore in artificially high values for the percentages cells in S and G_2M phases. The monoclonal antibody Ki-67 detects a nuclear antigen that is only present in proliferating cells. In an immunohistochemical study the growth fraction (number of cycling cells divided by the number of basal cells) of normal epidermis was calculated to be 5.2%[27]. This value approximates the percentage of cells in S and G_2M phases in the basal compartment of $5.5 \pm 0.9\%$ (Table 3).

Tape stripping, first described by Pinkus[21], provides a useful method for studying epidermal proliferation, differentiation and inflammation *in vivo*[1,7,11,19]. Using flow cytometry, we reconfirmed that maximal epidermal proliferation can be observed 44–48 h after removal of the stratum corneum. Proliferative activity was mainly located in

the basal compartment, but was also present in the suprabasal compartment, and, as expected, was not induced in the non-keratinocytes. After tape stripping we observed the proliferative activity of the basal cells to be increased by a factor of 8.5 compared with unchallenged skin. The decrease in keratin 10 expression (24 h) preceded any measurable change in DNA synthesis. Therefore, it is evident that the onset of keratinocyte differentiation does not imply a loss of proliferative activity as already suggested by Régnier et al.[26] and Bata-Csorga et al.[2]. Using an enzymological assay Chang et al. quantified epidermal polymorphonuclear leucocytes (PMNs) after tape stripping[8]. Maximal numbers of PMNs were observed 8 h after stripping. In the present study, however, clinical scores for erythema were maximal 36 h after tape stripping. Interestingly, maximal vimentin expression was observed after 44 h. At this time the percentage of vimentin-positive cells was 11.7%. In contrast to the situation in psoriasis, following tape stripping (without substantial changes in the total number of keratinocytes) the increase in vimentin-positive cells represents a relative increase as well as an absolute increase. These cells comprise Langerhans' cells, melanocytes and infiltrating cells such as monocytes, lymphocytes and PMNs. In inflamed skin the number of infiltrating cells can be approximated from the total number of vimentin-positive cells by subtracting the number of mesenchymal cells in normal epidermis (7.2%). In the tape stripping experiments the maximal number of infiltrating cells was 4.5%.

In conclusion, the combined approach of cell isolation with thermolysin–trypsin and multiparameter flow cytometry was shown to provide the optimal approach to analysing epidermal single-cell suspensions from normal and hyperproliferative skin. Different epidermal subpopulations could be discriminated and reliable cell cycle analysis performed. Furthermore, the characterization of epidermal cell suspensions using triple-labelling flow cytometry provided important information on the regeneration of the epidermis *in vivo* following tape stripping.

REFERENCES

1 Arnold WP, Kerkhof PCM van de The induction of epidermal ornithine decarboxylase following tape stripping is inhibited by a topical vitamin D_3 analogue (MC903). Br J Dermatol 1991, 125 6-8

2 Bata-Csorgo Z, Hammerberg C, Voorhees JJ, Cooper KD. Flow cytometric identification of proliferative subpopulations within normal human epidermis and the localization of the primary hyperproliferative population in psoriasis J Exp Med 1993, 178 1271-1281

3 Bauer FW Cell kinetics In Mier PD, Kerkhof PCM van de (eds) Textbook of psoriasis Churchill Livingstone, Edinburgh, 1986, pp 100-112
4 Bauer FW, Boezeman JBM Flow cytometric methods in human skin with respect to cell cycle kinetics In Wright NB, Camplejohn RS (eds) Psoriasis Cell Proliferation Churchill Livingstone, Edinburgh, 1983, pp 104-116
5 Bauer FW, Crombag NH, Grood RM de, Jongh GJ de Flow cytometry as a tool for the study of cell kinetics in epidermis Br J Dermatol 1980, 102 629-639
6 Boezeman JBM, Bauer FW, Grood RM de Flow cytometric analysis of the recruitment of G_0 cells in human epidermis in vivo following tape stripping Cell Tissue Kinet 1987, 20 99-107
7 Bokhoven MGJ van, Mare S de , Czarnetzki BM, Erp PEJ van, Boezeman JBM, Kerkhof PCM van de Active vitamin D_3 does not suppress recruitment of G_0 cells following injury Br J Dermatol 1988, 119 737-742
8 Chang A, Jongh GJ de, Mier PD, Kerkhof PCM van de Enzymatic quantification of polymorphonuclear leucocytes in normal and psoriatic skin following standardized injury Clin Exp Derm 1988, 13 62-66
9 Erp PEJ van, Rijzewijk JJ, Boezeman JBM, Leenders J, Mare S de, Schalkwijk J, Kerkhof PCM van de, Ramaekers FCS, Bauer FW Flow cytometric analysis of epidermal subpopulations from normal and psoriatic skin using monoclonal antibodies against intermediate filaments Am J Pathol 1989, 135 865-870
10 Germain L, Rouabhia M, Guignard R, Bouvard V, Auger A Improvement of human keratinocyte isolation and culture using thermolysin Burns 1993, 19 99-104
11 Gerritsen MJP, Erp PEJ van, Vlijmen-Willems IMJJ van, Lenders LTM, Kerkhof PCM van de Repeated tape stripping of normal skin a histological assessment and comparison with events seen in psoriasis Arch Dermatol Res 1994, 286 455-461
12 Glade CP, Erp PEJ van, Hooijdonk CAEM van, Elbers ME, Kerkhof PCM van de Topical treatment of psoriatic plaques with 1a,24 dihydroxyvitamin D_3 a multiparameter flow cytometrical analysis of epidermal growth, differentiation and inflammation Acta Derm Venereol (Stockh) 1995, 75 381-385
13 Gommans JM, Bergers M, Erp PEJ van, Hurk JJ van den, Mier PD, Roelfzema H Studies on the plasma membrane of normal and psoriatic keratinocytes I Preparation of material and morphological characterization Br J Dermatol 1979, 101 407-412
14 Hentzer B, Kobayashi T Dissociation of human adult epidermal cells by disulfide-reducing agents and subsequent trypsinization Acta Derm Venereol (Stockh) 1976, 56 19-25
15 Hooijdonk CAEM van, Glade CP, Erp PEJ van TO-PRO-3 iodide, a novel HeNe laser-excitable DNA stain as an alternative for propidium iodide in multiparameter flow cytometry Cytometry 1994, 17 185-189
16 Kiistala U, Mustakallio KK Dermo-epidermal separation with suction J Invest Dermatol 1976, 48 466-477
17 Kitano Y, Okada N Separation of the epidermal sheet by dispase Br J Dermatol 1983, 108 555-560

18. Longley J, Ding TG, Cuono C, Durden F, Crooks C, Hufeisen S, Eckert R, Wood GS Isolation, detection, and amplification of intact mRNA from dermatotome strips, epidermal sheets, and sorted epidermal cells. J Invest Dermatol 1991, 97: 974-979.
19 Mare S de, Erp PEJ van, Ramaekers FCS, Kerkhof PCM van de. Flow cytometric quantification of human epidermal cells expressing keratin 16 in vivo after standardized trauma Arch Dermatol Res 1990; 282: 126-130
20. Mason D, Gatter K. The role of immunochemistry in diagnostic pathology. J Clin Pathol 1987, 40: 1042-1054.
21. Pinkus H. Examination of the epidermis by the strip method of removing horny layers. II. Observations on thickness of the horny layer, and on mitotic activity after stripping. J Invest Dermatol 1951; 16: 383-386
22. Potten CS, Morris RJ. Epithelial stem cells in vivo. J Cell Sci (Suppl.) 1988; 10: 45-62.
23. Poumay Y, Boucher F, Degen A, Leloup R. Inhibition of basal cell proliferation during storage of detached cultured epidermal keratinocyte sheets. Acta Derm Venereol (Stockh) 1991; 71: 195-198.
24. Poumay Y, Roland IH, Leclercq-Smekens M, Leloup R. Basal detachment of the epidermis using dispase: tissue spatial organization and fate of integrin a6b4 and hemidesmosomes J Invest Dermatol 1994; 102. 111-117.
25. Raxworthy MJ, Cunliffe WJ, Wood EJ. The influence of proteases on the colony-forming efficiency of human keratinocytes in culture. Biochem Soc Trans 1987, 15: 519-520
26. Régnier M, Vaigot P, Darmon M, Pruniéras M. Onset of epidermal differentiation in rapidly proliferating basal keratinocytes. J Invest Dermatol 1986; 87: 472-476.
27. Rijzewijk JJ, Erp PEJ van, Bauer FW Two binding sites for Ki67 related to quiescent and cycling cells in human epidermis. Acta Derm Venereol (Stockh) 1989; 69 512-515
28. Smedts F, Ramaekers F, Robben H, Pruszczynski M, Muijen G van, Lane B, Leigh I, Vooijs P Changing patterns of keratin expression during progression of cervical intraepithelial neoplasia. Am J Pathol 1990; 136: 657-668.
29. Walzer C, Benathan M, Frenk E. Thermolysin treatment: a new method for dermo-epidermal separation. J Invest Dermatol 1989; 92: 78-81.
30 Willsteed EM, Bhogal BS, Das A, Bekir SS, Wojnarowska F, Black MM, Mckee PH An ultrastructural comparison of dermo-epidermal separation techniques. J Cutan Pathol 1991; 18: 8-12.

Chapter 3

IN VIVO MODELS TO STUDY THE DYNAMICS OF THE PSORIATIC LESION

3.1 The dynamics of the response of normal skin to single and multiple epicutaneous leukotriene B_4 applications analysed by three-colour flow cytometry

Conrad P. Glade, Rene J.G. Botermans, Piet E.J. van Erp, Peter C.M. van de Kerkhof, Department of Dermatology, University Hospital Nijmegen, The Netherlands

Acta Derm Venereol (Stockh) 1995; 75: 437-441

SUMMARY

Leukotriene B_4 (LTB_4) is a potent chemoattractant and a well-established stimulator of DNA-synthesis in keratinocytes. Previously, repeated applications of LTB_4 have been reported to induce a topically defined tachyphylaxis with respect to the extravasation of polymorphonuclear neutrophils. The aim of the present study was to quantify epidermal proliferation (% basal keratinocytes in S- and G_2M phase), epidermal keratinization (% keratin 10-positive keratinocytes) and the appearance of "non-keratinocytes", including melanocytes, Langerhans' cells and infiltrate cells (% vimentin-positive cells) in order to further elucidate the effect of chronic exposition of normal skin to LTB_4. Using three-colour flow cytometry, we could reconfirm that the response to one single epicutaneous application of LTB_4 was characterized by a marked increase of the percentage of basal keratinocytes in S- and G_2M phase, and a marked increase of non-keratinocytes. Repeated applications of LTB_4 induced a moderate increase of the percentage of cells in S- and G_2M phase and a moderate increase of the percentage of keratin 10-positive keratinocytes. Remarkably, the percentage of non-keratinocytes had decreased following repeated applications of LTB_4, compared to unchallenged normal skin. The present study suggests that chronic exposure of normal skin to LTB_4 induces changes which differ markedly from the histological features of the chronic psoriatic lesion. Therefore, LTB_4 is unlikely to be responsible for the perpetuation of the psoriatic plaque.

INTRODUCTION

The chemokine leukotriene B_4 (LTB_4), a metabolite from the 5-lipoxygenase pathway in the transformation of arachidonic acid, is a potent chemoattractant for polymorphonuclear neutrophils (PMNs) *in vitro* and *in vivo*[1,2]. PMNs, human keratinocytes *in vitro*[3] and human cells derived from epidermal biopsies[4] can synthesize LTB_4. It is well established that epicutaneous application of LTB_4 to normal skin causes a dose-dependent influx of leukocytes, subsequently followed by recruitment of cycling cells resulting in epidermal hyperproliferation[5-7]. As the main features of psoriasis consist of increased epidermal proliferation, cutaneous inflammation and impaired differentiation, it has been hypothesized that these changes in psoriasis might at least to some extent be induced by LTB_4. Indeed, compared to normal skin, there is a moderately increased LTB_4 synthesis in uninvolved skin of psoriatic patients[8]. In lesional psoriatic skin a massive overproduction of arachidonic acid metabolites, including LTB_4, has been demonstrated[9].

Until now the in vivo effect of LTB_4 has been assessed by qualitative analysis such as histology, immunocytochemistry or ultrastructural studies. Single application of LTB_4 to normal skin results in the induction of intra-epidermal neutrophil microabscesses after 24 h, which resolve after 48 to 72 h. Using elastase as a marker enzyme for PMNs, maximum elastase activity is measured 18 h after application of LTB_4[10]. Not only PMNs are attracted by epicutaneous LTB_4 application[6]. In the epidermis the number of T-lymphocytes reaches a maximum after 48 h. CD1a-positive Langerhans' cells show a moderate transition from the epidermis to the dermis after application of LTB_4. Also monocytes accompany PMN invasion[11]. A marked increase of cycling cells was demonstrated 72 h after application of LTB_4[6]. A flow cytometric quantification of DNA on unselected cell populations revealed an increase of the number of non-diploid cells from 3% to 15% 72 h after LTB_4 challenge[7]. No quantitative data are available on the effect of LTB_4 upon differentiation of keratinocytes.

Only to a limited extent can the response to single applications of LTB_4 be regarded as a model for psoriasis. So far, a single application of LTB_4 has never resulted in the appearance of a psoriatic lesion. The responses to repeated applications of LTB_4 have been evaluated by Wong et al.[12] using standard histology. Only modest histological changes, compared to unchallenged skin, have been reported by these authors. Indeed Colditz & Movat[13] also reported on the "habituation" to LTB_4 after repeated applications with respect to LTB_4-induced chemotaxis of PMNs.

The aim of the present study was to quantify aspects of epidermal proliferation, differentiation and inflammation in normal skin following single and repeated applications of LTB_4. A three-colour flow cytometric method[14-16] with simultaneous measurement of DNA content and the expression of two intermediate filament proteins was used. The following questions were addressed: i) to what extent are the flow cytometric analyses of epidermal cell populations compatible with the histological findings in skin biopsies taken after one single application of LTB_4?, and ii) what is the response of normal skin to repeated applications of LTB_4 compared to skin challenged with one single application of LTB_4 and unchallenged skin?

MATERIALS AND METHODS

Volunteers, LTB_4 applications and biopsy procedure

Nineteen healthy volunteers (9 males and 10 females, age range 20-29 years) without signs or history of skin diseases participated in this study after giving their informed consent. Aliquots of 100ng LTB_4 (Paesel GmbH, Frankfurt, Germany) dissolved in 10 µl ethanol were applied to the skin of the upper arm through a plastic cylinder obtained by cutting the edges from a micro test tube of 0.5ml (Eppendorf, Netheler Hinz GmbH, Hamburg, Germany). After evaporation of the ethanol under a stream of nitrogen gas, the test sites were marked with eosin and then occluded with impermeable dressings (Silverpatch, van der Bend bv, Brielle, The Netherlands) for 6 h.

Thirteen persons (6 males and 7 females) participated in the "single application study". At 0, 8, 24 , 32, 48, 72, 96 and 192 h after LTB_4 challenge, keratotome biopsies (0.2mm thick and 0.5cm^2) were taken using a small dermatome (Coriotome 6B333, Aesculap AG, Tuttlingen, Germany). The volunteers were divided at random with regard to the different time intervals, and three biopsies were obtained from each person. The 0-h test site was challenged with ethanol only. Before biopsies were taken, the clinical effects (erythema, induration, desquamation and pigmentation) were scored using a 4-point scale (0 = not present, 1 = slightly present, 2 = moderately present, 3 = markedly present). Histological studies were performed in four subjects at 24, 48 and 96 h after application of 100ng LTB_4. Routine haematoxylin-eosin (HE) staining was carried out on paraffin slides sectioned from 4-mm punch biopsies, taken after infiltration of the skin with 1% xylocain (1:100,000 adrenaline). At each time interval three biopsies were obtained. One biopsy was taken from non-challenged skin of one individual.

Six persons (3 males and 3 females) participated in the multiple application study. Every 24 h for 9 consecutive days (with a weekend break of 2 days after the 5th application) 100ng LTB_4 was applied to the same site of the skin of the upper arm. On day 9, 100ng LTB_4 in 10µl ethanol and 10µl ethanol only were applied to a second and to a third test site, respectively. Twenty-four hours after these last applications in each volunteer three keratotome biopsies were taken from these test sites, as described before. Clinical scores were assessed immediately before taking the biopsies.

Cell isolation procedure

Epidermal single cell suspensions were prepared as described before[16]. In short, the biopsies were incubated in phosphate-buffered saline (PBS) containing 0.25mg/ml trypsin (Sigma, St. Louis, USA) and 3.0mg/ml dithioerythritol (Sigma) for 30 min at 37°C. Then, in PBS containing 10% heat-inactivated newborn calf serum (HINCS, Life Technologies Ltd., Paisley, UK) the dermis was separated from the epidermis with fine forceps. The remaining epidermis was gently mixed on a vortex to loosen the keratinocytes, resulting in a single cell suspension. After the horny layer had been discarded, the suspension was centrifuged, the supernatant removed and the cells fixed in 70% ice-cold ethanol. The cell suspension was stored at -20°C until staining and flow cytometric analysis.

Staining procedure

A triple labelling was performed, using a DNA fluorochrome combined with two antibodies against intermediate filaments. Samples of the cell suspensions, containing approximately 1-2 × 10^5 cells, were stained. The procedure has been described by us in detail before[16]. To assess hyperproliferation, the DNA fluorochrome TO-PRO-3 iodide (TP3, Molecular Probes, Eugene, USA) was used[15]. TP3 intercalates with double-string DNA and permits measurement of the proliferative activity of cells by quantification of the percentage of cells in S- and G_2M phase. As TP3 also binds to RNA to some extent, it was used in combination with RNase. To study the inflammatory response we used Vim3B4 (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK). This IgG2a-type mouse monoclonal antibody stains vimentin, an intermediate filament-type protein which occurs in mesenchymal cells[17]. In this study, PMNs, lymphocytes, monocytes, macrophages, melanocytes and Langerhans' cells were stained by Vim3B4. To quantify the effect of topical LTB_4 application on the differentiation process, we used the IgG1-type mouse monoclonal antibody RKSE60

(Dept. of Mol. Biology, University of Maastricht, The Netherlands). RKSE60 is directed against keratin 10, an intermediate filament-type protein that is expressed in differentiating keratinocytes[18]. Three-colour fluorescence was obtained with the fluorochromes fluorescein-isothiocyanate (FITC) and phycoerythrin (PE), which were conjugated to monoclonal goat antibodies against mouse IgG2a and mouse IgG1, respectively (Southern Biotechnology Associates, Birmingham, USA), in combination with TP3.

Flow cytometric analysis

In a dual laser system (Epics® Elite flow cytometer, Coulter, Luton, UK) the fluorochromes FITC and PE were excited at 488nm (air-cooled argon ion laser, 15mW) and TP3 at 633nm (HeNe laser, 10mW). Emission spectra were separated with bandpass filters of 525 ± 10nm, 575 ± 10nm and 675 ± 10nm, for FITC (green signal), PE (orange signal) and TP3 (red signal), respectively. The spectral overlap from PE to TP3 was 2% and therefore easily compensated electronically. Compensation for FITC in PE was 21%. After correction for clumps and cellular debris by setting appropriate gates, 5,000 cells from each sample were analysed. With Elite and Multicycle™ software percentages of vimentin- and keratin 10-positive cells and percentage of cells in S- and G_2M phase were calculated. As this triple labelling procedure allows simultaneous assessment of different epidermal cell populations, we were able to quantify the proliferative activity of the basal epidermal compartment exclusively.

RESULTS

Single application

The clinical scores at different time intervals after one single LTB_4 application are shown in Fig. 1. Maximal erythema was reached after 48 h. Maximal induration occurred 32 h after LTB_4 challenge. Desquamation and pigmentation appeared at 48 h and 72 h respectively, and increased slowly, reaching a maximum at 192 h at all test sites.

The histological assessment of the response to epicutaneous application of LTB_4 was in line with previous reports[5,6]. A massive infiltration with PMNs was observed 24 h after challenge. PMNs were partly grouped together as abscesses and partly scattered through the epidermis. In the dermis the infiltrate was pronounced at perivascular and subepidermal localizations. At 48 h no microabscesses were present in the epidermis,

but some PMNs could be observed. Between the stratum corneum and the stratum spinosum an amorphous zone with a granular aspect was identified, and focal parakeratosis was observed. A mononuclear dermal infiltrate was seen. At 96 h the picture was comparable to the situation after 48 h. No PMNs were seen in the epidermis and the dermal infiltrate was mainly located in the perivascular areas.

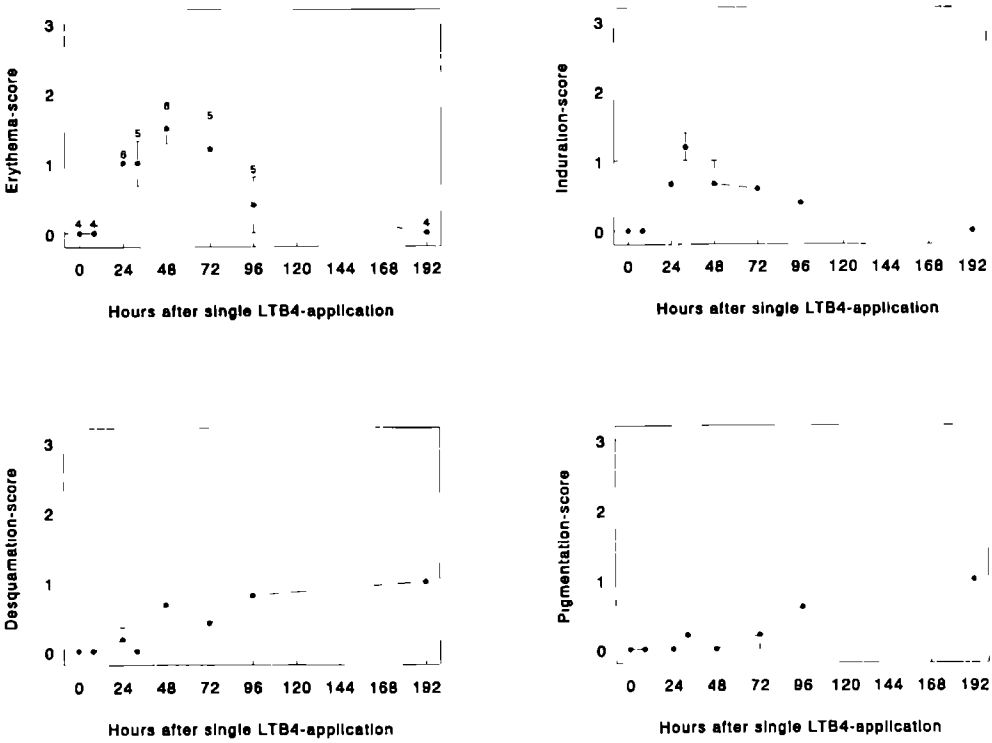


Figure 1. Clinical scores (mean ± SEM) at different time intervals after a single application of 100 ng LTB_4. (0 = not present, 1 = slightly present, 2 = moderately present, 3 = markedly present). The number of observations is indicated above the error bars.

Fig. 2 shows the results of the flow cytometric analysis. In unchallenged normal skin the percentage of vimentin-positive cells was 7.5% ± 1.1% (mean ± SEM). After 48 h the maximum of 11.2% was reached. The amount of non-keratinocytes then returned to levels of ± 5.3%. In the unchallenged skin, the percentage of keratin 10-positive cells, i.e. differentiated keratinocytes, was 61.5% ± 4.5%. After a slight decrease the percentage returned to the normal level from 96 h onwards. The percentage of basal

keratinocytes in S- and G_2M phase in the unchallenged skin was 8.8% ± 0.8%. This percentage remained more or less stable up to 48 h. From 48 h onwards there was a statistically significant increase of basal cells in S- and G_2M phase with a maximum of 21.2% at 72 h (see Table 1), showing a tendency to return to normal at 192 h.

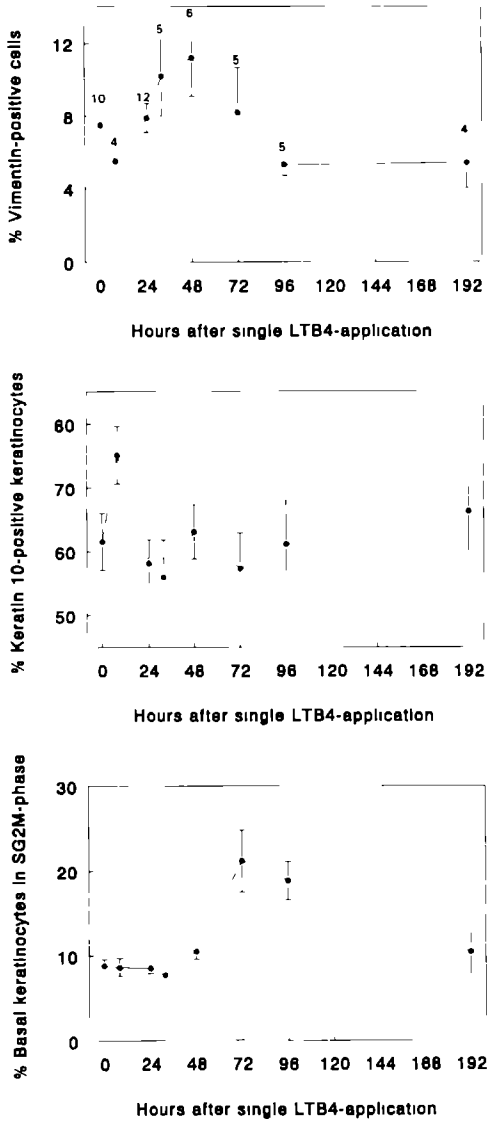


Figure 2. Flow cytometric analysis (mean ± SEM) of epidermal cell suspensions prepared from biopsies taken from normal skin at different time intervals after a single application of 100 ng LTB_4. The number of biopsies is indicated above the error bars.

Table 1. Comparison of flow cytometric values (mean ± SEM) in unchallenged normal skin, in normal skin after single (peak values) and multiple applications of 100 ng LTB_4, and in psoriatic skin

	Normal skin (n=10)	Single application (n=5/6)	Multiple application (n=6)	Psoriatic skin (n=38)
%S- and G_2M (basal keratinocytes)	8 8 ± 0 8	21 2 ± 3 7[a]	12 2 ± 1 9[b d]	18 9 ± 1 3
%vimentin-positive cells	7 5 ± 1 1	11 2 ± 2 1	4 6 ± 1 4[c d]	15 6 ± 1 7
%keratin 10-positive keratinocytes	61 5 ± 4 5	57 3 ± 5 6	68 7 ± 6 1[c]	36 5 ± 2 3

[a] p<0 05, versus normal skin (unequal variance t-test)

[b] p<0 01, versus normal skin (paired t-test, n=6)

[c] p<0 05, versus normal skin (paired t-test, n=6)

[d] p<0.05, versus single application (unequal variance t-test)

Multiple application

The clinical response to multiple applications was comparable to the response to one single application. Both erythema and induration were maximal at 72 h. From 72 h onwards a sharp decline of these clinical scores was observed Both desquamation and pigmentation slowly increased until 264 h.

In Table 1 the flow cytometric data of normal unchallenged skin and normal skin after single and multiple applications are summarized. Statistical analysis revealed a significant increase with respect to keratin 10-expression and proliferative activity, and a significant decrease with respect to vimentin expression for multiple challenged skin compared to unchallenged skin. The mean percentage of vimentin-positive cells in the multiple application sites (4.6%) was significantly lower than the peak value at 48 h in the single application series. The percentage of basal keratinocytes with a more than diploid DNA content was significantly lower in multiple challenged sites (12.2%) than the peak value at 72 h after single application.

DISCUSSION

In previous flow cytometric studies, mean percentages of cells in S- and G_2M phase of 2.9% and 4.0% have been measured in normal skin[7,14]. These values refer to the proliferative activity of the total cell population present in the cell suspension. The value of 8.8% in unchallenged skin (present study), however, reflects the specific proliferative activity of the basal keratinocytes and represents a value of 3.1% for the

total cell population. Percentages of 57.2 for keratin 10-positive cells and 7.6 for vimentin-positive cells were found in healthy skin[14]. Correction for vimentin-positive cells reveals a percentage of 61.9% of keratin 10-positive keratinocytes. As these values correspond with the present data, the reproducibility of the flow cytometric method is warranted.

Using histological and flow cytometric analysis we reconfirmed that epicutaneous application of one single dose of 100ng of LTB_4 consistently yields an inflammatory response. A single application of 100ng LTB_4 to normal skin resulted in a thin layer of parakeratosis after 48 h[5]. Repeated intracutaneous injection of LTB_4 in guinea pig ears caused epidermal hyperplasia with an unaltered keratinization pattern[19]. The tendency of a temporary decrease of keratin 10-positive keratinocytes after single application of 100ng LTB_4 to human skin and the appearance of parakeratosis suggest that *in vivo* LTB_4 might interfere with the process of keratinization. The present data show that there is an increase in epidermal proliferation following single application of LTB_4 (Fig. 2). Other studies confirm the proliferative response 72-96 h after LTB_4 application[6,7]. Although a direct effect of LTB_4 on the germinative cell population has been described *in vitro*[20], an indirect effect via interferences with the suprabasal compartment or via the inflammatory response is by no means ruled out.

Maximal erythema was seen 48 h after single-dose LTB_4 application. Interestingly, the number of vimentin-positive cells also reached a maximum 48 h after challenge. Histological studies have demonstrated that the infiltrate mainly consists of PMNs during the first 24 h. However, the maximum percentage of vimentin-positive cells at 48 h after the application of LTB_4 is not exclusively due to high levels of PMN at this time interval but is mainly caused by infiltrating T-lymphocytes reaching their maximum after 48 h[5,6]. Also monocytes invade the epidermis after LTB_4 challenge[11]. It is remotely possible that not all PMNs present in the epidermis were analysed flow cytometrically. A certain quantity of PMNs might have been captured in the stratum corneum and may therefore not reach the cell suspension. Furthermore, some PMNs degenerate at the moment of extravasation, resulting in pycnotic nuclei and vacuolisation[11].

During the first 3 days, the clinical response to single and repeated applications of LTB_4 proved to be similar. However, during the chronic LTB_4 exposure the erythema began to subside from 72 h onwards, in spite of the five following applications. This response is compatible with the "tachyphylaxis" of the LTB_4 induced accumulation of PMNs, as reported by Wong et al. and by Colditz & Movat[12,13]. Besides a

quantification of epidermal proliferation, percentage of vimentin-positive cells and percentage of keratin 10-positive keratinocytes in unchallenged and LTB_4-challenged normal skin, Table I provides these data in psoriatic skin without epicutaneous LTB_4 challenge[16]. It can be concluded that (i) compared to unchallenged skin (intra-individual comparison), repeated applications of LTB_4 induce a decrease in the number of vimentin-positive cells, an increase of keratin 10-positive keratinocytes, and an increase of epidermal proliferation;. (ii) compared to the maximum values reached by one single application LTB_4 (inter-individual comparison), a reduced responsiveness was observed in the repeatedly challenged skin with respect to the number of vimentin-positive cells and epidermal proliferation; and (iii) comparing the present data with observations in the psoriatic plaques (without epicutaneous LTB_4 challenge), multiple applications of LTB_4 induce far less hyperproliferation, far less intra-epidermal accumulation of vimentin-positive cells and not a reduction but an increase of the number of keratin 10-positive keratinocytes.

In conclusion, the present data indicate that LTB_4 cannot be a major factor in the perpetuation of epidermal proliferation and inflammation, and the reduction of keratin 10 expression in the chronic psoriatic lesion. However, in an early stage of the psoriatic process, changes similar to one single application might be of pathogenetic significance.

REFERENCES

1 Ford-Hutchinson AW, Bray MA, Doig MV, Shipley ME, Smith MJH Leukotriene B_4, a potent chemokinetic and aggregating substance released from polymorphonuclear leucocytes Nature 1980, 286 264-265

2 Soter NA, Lewis RA, Corey EY, Austen KF Local effects of synthetic leukotrienes (LTC_4, LTD_4, LTE_4 and LTB_4) in human skin J Invest Dermatol 1983, 80 115-119

3 Brain SD, Camp RD, Leigh IM, Ford-Hutchinson AW The synthesis of leukotriene B_4-like material by cultured human keratinocytes J Invest Dermatol 1982, 78 328

4 Grabbe J, Rosenbach T, Czarnetzki BM Production of LTB_4-like chemotactic arachidonate metabolites from human keratinocytes J Invest Dermatol 1985, 85 527-530

5 Camp R, Russel-Jones R, Brain S, Woollard P, Greaves M Production of intraepidermal microabscesses by topical application of leukotriene B_4 J Invest Dermatol 1984, 82 202-204

6 de Jong EMGJ, van Erp PEJ, van Vlijmen IMJJ, van de Kerkhof PCM The inter-relation between inflammation and epidermal proliferation in normal skin following epicutaneous application of leukotriene-B_4, an immunohistochemical study Clin Exp Dermatol 1992, 17 413-420

7 Bauer FW, van de Kerkhof PCM, Maassen-de Grood RM Epidermal hyperproliferation following the induction of microabscesses by leukotriene B_4 Br J Dermatol 1986, 114 409-412
8 Ziboh VA, Casebolt TL, Marcelo CL, Voorhees JJ Biosynthesis of lipoxygenase products by enzyme preparations from normal and psoriatic skin J Invest Dermatol 1984, 83 426-430
9 Brain S, Camp R, Dowd P, Kobza-Black A, Greaves M The release of leukotriene B_4-like material in biologically active amounts from the lesional skin of patients with psoriasis J Invest Dermatol 1984, 83 70-73
10 Lammers AM, van de Kerkhof PCM Response of polymorphonuclear leucocytes to topical leukotriene B_4 in healthy and psoriatic skin Br J Dermatol 1987, 116 521-524
11 van de Kerkhof PCM, Copius Peereboom-Stegeman JHJ, Boeijen J An ultrastructural study of the response of normal skin to epicutaneous application of leukotriene B_4 J Dermatol 1991, 18 271-278
12 Wong E, Camp RD, Greaves MW The responses of normal and psoriatic skin to single and multiple topical applications of leukotriene B_4 J Invest Dermatol 1984, 84 421-423
13 Colditz IG, Movat HZ Desensitization of acute inflammatory lesions to chemotaxins and endotoxin J Immunol 1984, 133 2163-2168
14 van Erp PEJ, Rijzewijk JJ, Boezeman JBM, Leenders J, de Mare S, Schalkwijk J, et al Flow cytometric analysis of epidermal subpopulations from normal and psoriatic skin using monoclonal antibodies against intermediate filaments Am J Pathol 1989, 135 865-870
15 van Hooijdonk CAEM, Glade CP, van Erp PEJ TO-PRO-3 iodide, a novel HeNe laser-excitable DNA stain as an alternative for propidium iodide in multiparameter flow cytometry Cytometry 1994, 17 185-189
16 Glade CP, van Erp PEJ, van Hooijdonk CAEM, Elbers ME, van de Kerkhof PCM Topical treatment of psoriatic plaques with 1α,24 dihydroxyvitamin D_3 a multiparameter flow cytometrical analysis of epidermal growth, differentiation and inflammation Acta Derm Venereol (Stockh) 1995, 75 381-385
17 Bauer FW, Boezeman JB, van Engelen L, de Grood RM, Ramaekers FC Monoclonal antibodies for epidermal population analysis J Invest Dermatol 1986, 87 72-75
18 Ramaekers FC, Puts JJ, Moesker O, Kant A, Huysmans A, Haag D, et al Antibodies to intermediate filament proteins in the immunohistochemical identification of human tumours an overview Histochem J 1983, 15 691-713
19 Ruzicka T, Burg G Effects of chronic intracutaneous administration of arachidonic acid and its metabolites Induction of leucocytoclastic vasculitis by leukotriene B_4 and 12-hydroxyeicosatetraenoic acid and its prevention by prostaglandin E_2 J Invest Dermatol 1987, 88 120-123
20 Kragballe K, Desjarlais L, Voorhees JJ Leukotrienes B_4, C_4 and D_4 stimulate DNA synthesis in cultured human epidermal keratinocytes Br J Dermatol 1985, 113 43-52

3.2 A clinical and flow cytometric validation of a model to study remission and relapse in psoriasis

Conrad P. Glade[1], Piet E.J. van Erp[1], Hildburg Werner-Schlenzka[2], Peter C.M.van de Kerkhof[1], [1]Department of Dermatology, University Hospital Nijmegen, The Netherlands and [2]Clinical Development, Center of Dermatology, Schering AG, Berlin, Germany

submitted

SUMMARY

Psoriasis is a chronic disease with remissions and relapses. Aim of the present study was to develop and evaluate a model to analyse remission and relapse characteristics in psoriasis vulgaris after successful treatment with clobetasone propionate under different experimental conditions: alcoholic solution under occlusion, occlusion and non-treatment. Fifteen patients participated in the study. The study was divided into a clinical and flow cytometric evaluation. They were performed on two different psoriatic lesions in the same patient. All lesions were treated with clobetasone propionate up to a maximum of 23 days. Only cleared lesions were investigated as follows. For the clinical evaluation the area of the cleared lesion was divided into three test sectors: (1) was treated daily with an alcoholic solution under occlusion, (2) was treated with occlusion and (3) was left untreated for the observation period of 28 days. For the flow cytometric evaluation, the healed lesion was left completely untreated and unoccluded for the observation period of 35 days and biopsies were taken (i) before the start of the clobetasone propionate therapy, (ii) at clearance, (iii) at relapse from pinpoint lesions and (iv) at relapse from the visibly unaffected skin of the cleared lesion. Multiparameter flow cytometric analysis with markers for epidermal proliferation (% basal keratinocytes in S- and G_2M phase), differentiation (% keratin 10 positive keratinocytes) and inflammation (% vimentin positive cells) was performed.

The clinical evaluation scheme worked well and was able to discriminate between the different therapy modalities. At the end of the clinical evaluation 30% of sector 1 showed characteristic features of a relapse, 50% of sector 2 and 80% of sector 3. The results demonstrate that occlusion has an inhibitory effect on the tendency to relapse

after successful treatment with clobetasone propionate. This tendency was enhanced by the alcoholic solution applied.

The flow cytometric analysis showed at clearance a very low proliferative activity of the basal compartment. This activity increased in the visibly unaffected skin of the healed lesion at the time of relapse and was highest in the pinpoint lesions. Interestingly, at relapse the proliferative activity in the suprabasal compartment of the visibly unaffected skin had increased to values identical to the pinpoint lesions.

The present model allows standardized comparison of different approaches for maintenance therapy in psoriasis vulgaris. Quantitative information on these processes can be obtained by multiparameter flow cytometry.

INTRODUCTION

Maintenance treatment is an important approach in patients with fast relapsing psoriasis. Several authors have indicated that early psoriatic lesions differ from the chronic established psoriatic plaque[1]. In acute psoriatic lesions polymorphonuclear leukocytes, monocytes and mast cells are a dominating feature in contrast to a more modest appearance of these cells in the chronic plaque[2,3]. Whereas the advancing edge of the psoriatic plaque does not yet contain skin associated antileukoproteinase, the central zone of the psoriatic does[4]. The therapeutic relevance of the distinction of the acute and chronic stage of the psoriatic lesion is indicated by the observation that acitretin monotherapy is poorly effective in chronic plaque psoriasis but very effective in acute instable psoriasis[5].

Before the therapeutic responsiveness of acute relapsing psoriasis can be studied, approaches to quantify characteristics of relapsing psoriasis are mandatory. The aim of the present study was to provide a standardized model for analysis of relapse following corticosteroid induced clearing of a psoriatic lesion. This model was validated by a clinical study of the relapse characteristics under three post-therapy conditions: (1) daily application of an alcoholic solution under occlusion, (2) occlusion and (3) left untreated for the observation period of 28 days. In particular the following questions were addressed: what are the dynamics of reoccurrence of erythema, induration and desquamation under each of the above-mentioned post-therapy conditions, using a semiquantitative 5-point scale? Can a relapse clearly be defined by clinical parameters and can the clinical evaluation be used to differentiate between the different post-therapy conditions?

An alcoholic solution was chosen in this study in preparation for a clinical study with a specific active agent to be administered in an alcoholic solution.

In addition to the clinical validation in 15 lesions, a flow cytometric study of relapse characteristics was performed in a further 13 lesions in the same patients. The conditions for the lesions used for the flow cytometric evaluation were the same as for sector (3) in the clinical evaluation: after clearing the lesion with clobetasol it was left unoccluded and untreated. Biopsies were taken: (i) before the start of clobetasol therapy, (ii) at clearance, (iii) at relapse from pinpoint lesions, and (iv) at relapse from the visibly unaffected skin of the cleared lesion. Using a multiparameter technique with simultaneous assessment of epidermal DNA content (TO-PRO-3 iodide) and expression of intermediate filaments keratin 10 and vimentin we were able to measure the dynamics of changes of quantitative parameters for epidermal proliferation, differentiation and inflammation during clearance and relapse[6-8]. As flow cytometry was performed on epidermal cell suspensions prepared from 3-mm punch biopsies these cell biological aspects could be measured in both pinpoint lesions and visibly unaffected skin of the same lesion simultaneously.

MATERIALS AND METHODS

Subjects and test lesions

Male and female patients over 18 years with chronic plaque psoriasis were recruited after written informed consent was obtained. The patient was to have at least two lesions with a minimal size of 10 cm^2 not directly located above the elbow or knee. The score for erythema, thickness, and scaling had to be at least grade 2 (assessment on a 5-point-scale: 0 = not present, 1 = mild, 2 = moderate, 3 = severe, 4 = very severe). Exclusion criteria were pregnancy or lactation and known allergy to the test medications: clobetasone 17-propionate, the alcoholic solution or to the hydroactive dressing. Systemic treatment with antipsoriatic or immunosuppressive drugs and UVB therapy were not allowed.

A clinical test lesion and a flow cytometric test lesion were selected in each patient. Both lesions were treated twice daily with clobetasone 17-propionate lotion (Glaxo Wellcome, Nieuwegein, The Netherlands) under hydroactive occlusion (Cutinova®Thin, Beiersdorf, Hamburg, Germany) until clearance or for maximally 23 days. Clearance was defined as no induration, no desquamation and no or only a mild

erythema (grade 1). Patients whose lesions did not clear by day 23 were excluded from further participation.

Clinical study

The study design was an open intraindividual comparison. For the clinical evaluation, 3 sectors - each 1.8 cm^2 in size - were marked in the area of the healed test lesion. Sector 1 was treated once daily with an alcoholic solution (Schering AG, Berlin, Germany) containing 5.0 g isopropylmyristat and 74.0 g isopropanolol in 100 ml solution under hydroactive occlusion, sector 2 with hydroactive occlusion and sector 3 was left untreated and unoccluded. Patients visited the department on day 7, 11, 14, 17, 20, 23, 26, and 29 (end of study) after clearance for evaluation of clinical scores, itching, burning and adverse events. Relapse was defined as an at least grade 2 erythema with additionally at least grade 2 thickness and/or scaling. Photographic documentation was performed at recruitment, at clearance, at relapse and at the end of study.

Flow cytometric study

From the test lesion chosen for the flow cytometric study, 3-mm punch biopsies were obtained (i) before treatment with clobetasone 17-propionate, (ii) at clearance, (iii) at relapse from pinpoint lesions and (iv) from the visually unaffected skin of the former lesion for flow cytometric analysis. After healing the cleared lesion was left untreated and unoccluded and observed for a period of 35 days. The observation period in the flow cytometric study was one week longer compared to the clinical study in order to permit a further development of initial psoriatic lesions in this experimental group. Relapse was defined as the clinical occurrence of pinpoint lesions in the area of the cleared lesion. Clinical scores were assessed before treatment and at clearance.

Epidermal single cell suspensions were prepared shortly after biopsying using the combined thermolysin-trypsin separation method as described previously[9] and stored in 70% ethanol at -20°C until staining. Approximately $1\text{-}2x10^5$ cells were simultaneously labelled with the DNA fluorochrome TO-PRO-3 iodide (TP3, Molecular Probes, Eugene, USA) and mouse antibodies directed against intermediate filaments vimentin (Vim3B4, IgG_{2a}, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) and keratin 10 (RKSE60, IgG_1, Dept. of Mol. Biology, University of Maastricht, The Netherlands). Emitting fluorochromes were TP3 in combination with fluorescein-isothiocyanate (FITC) and phycoerythrin (PE), which were conjugated to

subtype-specific monoclonal goat antibodies against mouse IgG_{2a} and mouse IgG_1, respectively (Southern Biotechnology Associates, Birmingham, USA).
From each sample 5,000-10,000 gated cells were measured and analysed using an EPICS® Elite flow cytometer (Coulter, Luton, UK) equipped with a dual laser system. PE and FITC were excited with an air-cooled argon ion laser (15mW, 488nm). TP3 was excited with a HeNe laser (10mW, 633nm). Fluorescence was measured using bandpass filters of 520-530nm (green, FITC), 555-595nm (orange, PE), and 670-680nm (red, TP3). The area/peak ratio of the red signal (DNA) was used to discriminate between doublets of diploid cells (clumps) and real single tetraploid cells[10]. After setting appropriate gates with the EPICS® Elite software percentages of vimentin- and keratin 10 positive cells were calculated. Using Multicycle™ software (Phoenix Flow Systems, San Diego, USA) the percentages of basal (vimentin negative and keratin 10 negative) and suprabasal keratinocytes (vimentin negative and keratin 10 positive) in S- and G_2M phase (proliferation) of the cell cycle were calculated from DNA histograms.

Statistical analysis

Paired values were analysed with the paired two-sample t-test for means. For unpaired data the two-sample t-test assuming equal variances was used. With ANOVA (single-factor) durations of remission were compared. ANOVA (two-factor) and Duncans multiple range test was performed to analyse clinical scores.

RESULTS

Fifteen patients were recruited, 9 male and 6 female. All of them were included in the safety analysis. The 15 patients had a mean age of 53.9 ± 9.5 years (mean ± SD). The mean duration of disease was about 20 years with a minimum of 4 years and a maximum of nearly 60 years.

Clinical study

In 10 out of the 15 patients the test lesions could be cleared within the 23 days allowed for the clobetasone propionate treatment. The mean clearing time was 16.4 ± 1.8 days (range 8-23 days). The cleared lesion was divided into three sectors and monitored over a period of 28 days. One patient did not appear for observation after day 23. This patient had a relapse in all 3 sectors on day 11 and the results were included in the

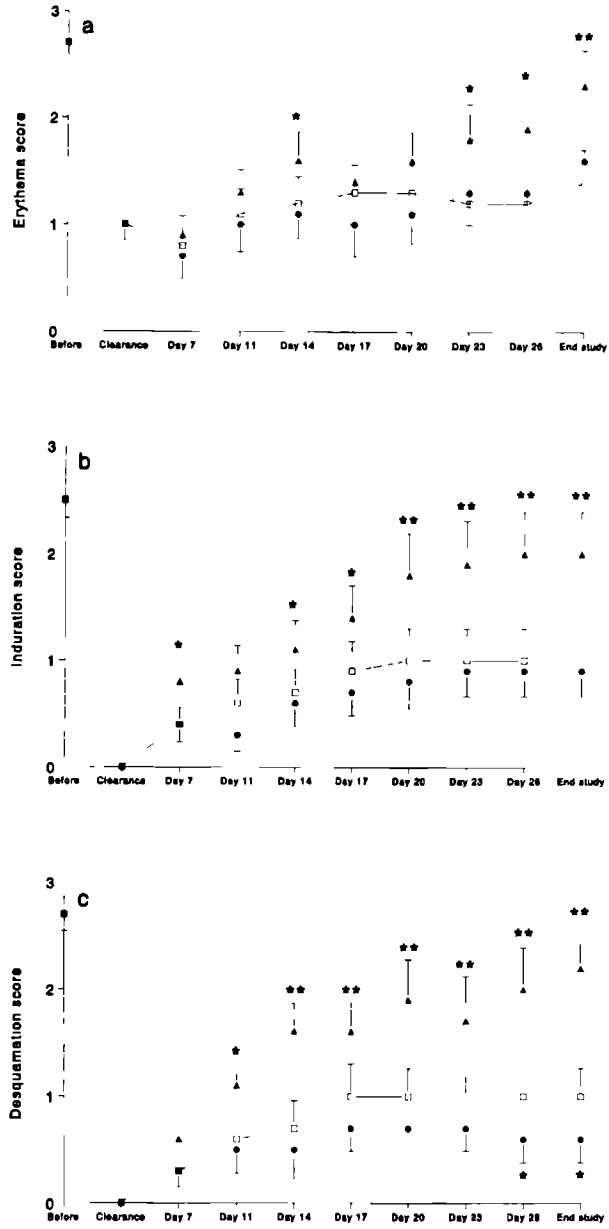


Figure 1a-c. Clinical scores (mean ± SEM) assessed on a 5-point scale (0=not present, 1=mild, 2=moderate, 3=severe, 4=very severe) for erythema (**a**), induration (**b**), and desquamation (**c**) of 10 psoriatic lesions during treatment- and remission period at different test sectors: sector 1, alcoholic solution and occlusion (●), sector 2, hydroactive occlusion only (□): sector 3, no occlusion (▲). Significance is expressed as compared to occlusion only: * ($P < 0.05$) and ** ($P < 0.01$)

evaluation. In the observation period of 28 days the relapse percentage was 30% (3 patients) in sector 1 - alcoholic solution with occlusion - ; 50% (5 patients) in sector 2 - occlusion only - and 80% (8 patients) in sector 3 - no treatment. Intraindividual comparison of the three sectors showed that relapse always occurred earlier or at the same time in sector 3 compared to sector 2 and earlier or at the same time in sector 2 compared to sector 1.

Fig. 1a-c summarizes the assessment of clinical scores for erythema, induration and desquamation during the study. It can be seen that during the observation period of 28 days for all clinical scores significant differences developed between occluded and non-occluded areas. Occlusion has an inhibitory effect on erythema, induration and desquamation. However, the effect is more pronounced with respect to induration and desquamation compared to erythema. Comparing the alcoholic solution under occlusion with occlusion alone, there is only a significant difference for the desquamation score at the end of the study.

Flow cytometric study

Thirteen of 15 patients participated in the flow cytometric study. One patient preferred not to be biopsied, in the other patient no suitable test area was left for the flow cytometric study. In 11 of the 13 test lesions clearance was reached (mean time 18.1 ± 1.9 days; mean ± SEM). In the observation period of 35 days 7 (64%) of the 11 cleared test lesions relapsed. The mean time until relapse was 17.9 ± 4.4 days (mean ± SEM). Although the durations of the remission periods of the untreated test lesions from both the clinical and the flow cytometric study differed substantially in some patients (up to 23 days) no significant differences for the whole groups existed (ANOVA single-factor analysis).

The results of the flow cytometric analyses on 36 samples are summarized in Fig. 2a-d. At the beginning before treatment with clobetasone propionate the psoriatic lesions were characterized by a high percentage of vimentin positive cells, a low number of keratin 10 positive keratinocytes and a high number of basal keratinocytes in S- and G_2M phase. At clearance (n = 11) a marked and significant improvement of all markers was found. In the basal and the suprabasal compartment proliferation was substantially decreased.

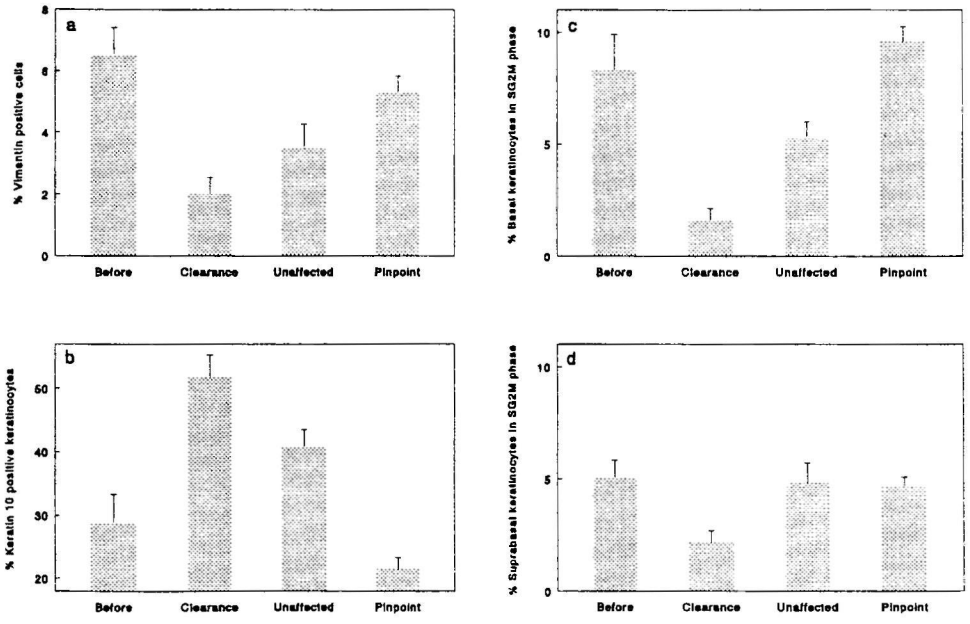


Figure 2a-d. Flow cytometric analysis (mean ± SEM) of markers for % vimentin positive cells (**a**), % keratin 10 positive keratinocytes (**b**), and % keratinocytes in S- and G_2M phase in basal (**c**) and suprabasal (**d**) compartments

At relapse (n = 7) from each test lesion 2 biopsies were obtained: one from the pinpoint lesions and one from the visibly unaffected skin of the cleared lesion. The flow cytometric analysis of the pinpoint lesions revealed a return to the psoriatic phenotype for all markers. No significant differences existed between the psoriatic lesions before treatment and the pinpoint papules at relapse. Significant differences were observed between the percentage of keratin 10 positive keratinocytes at clearance and the visibly unaffected skin of the cleared lesion at relapse ($P < 0.05$). In the visibly unaffected skin of the cleared lesion at relapse the percentage of keratinocytes in S- and G_2M phase was significantly higher compared to clearance in the basal ($P < 0.01$) and in the suprabasal ($P < 0.05$) compartment. Also with respect to the relative number of vimentin positive cells in the visibly unaffected skin of the cleared lesion at relapse showed values that were intermediate between the situation at clearance (N.S.) and relapsed skin ($P < 0.05$). All flow cytometric parameters including the proliferative

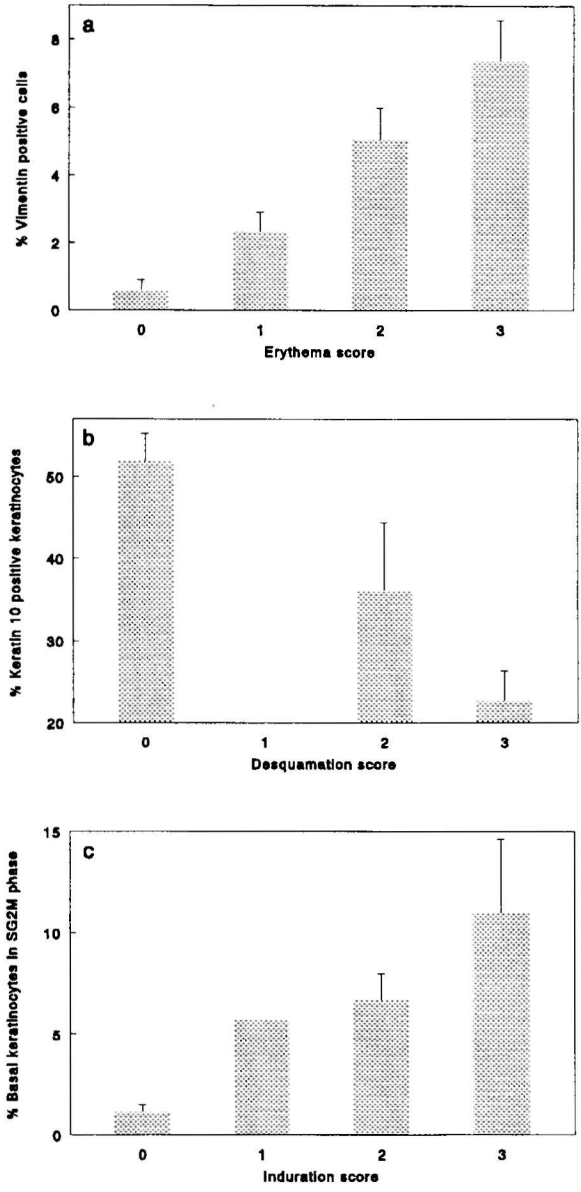


Figure 3a-c. Correlation between erythema and percentages of vimentin positive cells (**a**), induration and percentages of basal keratinocytes in S- and G_2M phase (**b**), and desquamation and percentages of keratin 10 positive keratinocytes (**c**) in psoriatic lesions before and after clearance with clobetasol propionate lotion under hydroactive occlusion

activity in the basal compartment were significantly different between the involved and the visibly unaffected skin of the cleared lesion at relapse. Remarkably, at relapse the proliferative activity in the suprabasal compartment in both the pinpoint and the visibly unaffected skin was comparable. In Fig. 3 we compared clinical features of inflammation (erythema), impaired differentiation (desquamation) and hyperproliferation (induration) with the cell biological markers of these processes. It can be seen that in all cases a strong correlation exists between clinical and flow cytometric parameters (Fig. 3a, erythema score versus % vimentin positive cells: $r = 0.76$, $P < 0.0001$; Fig. 3b, desquamation score versus % keratin 10 positive keratinocytes: $r = -0.73$, $P < 0.0001$; Fig. 3c, induration score versus % basal keratinocytes in S- and G_2M phase: $r = 0.74$, $P < 0.0001$).

DISCUSSION

In the present study we provide a model to study the dynamics of the fast relapsing psoriatic lesion after successful treatment with clobetasone propionate applied twice daily under occlusion. In the clinical part of the study, three different post-treatment modalities were compared on different sectors of the cleared lesion and observed for 28 days. From a second lesion of the same patient biopsies were taken before clobetasol treatment, at clearance, and at relapse from pinpoint lesions and from the visibly unaffected skin. Flow cytometric analysis of the samples permitted quantitative measurement of the epidermal changes with respect to proliferation, differentiation and inflammation.

Defining clinical clearance as maximally grade 1 erythema, no induration and no desquamation, we observed a mean clearing time of 17.3 days (range 7-28 days, $n = 21$). Other studies describe clinical healing after 6-10 days[3] and 12 days[11] after treatment with clobetasol propionate under occlusive dressings. In one study[3], however, clearance criteria allowed a grade 3 erythema and in the other study[11] these criteria were not mentioned. In most studies the clearance percentage after treatment with clobetasone propionate under occlusive dressings has been reported to be 100%. In contrast, the present study revealed a percentage of clearing in only 75% after 3 weeks of treatment with clobetasone propionate twice daily under occlusive dressings.

All markers for epidermal proliferation, differentiation and inflammation significantly decreased during remission induction. Epidermal proliferation was even totally blocked in some patients as also shown by Goodwin *et al.*[12]. This implies that the

situation at clearance was substantially different from the situation before therapy and constituted a suitable starting point to study the process of change towards the psoriatic phenotype. In comparison to treatment with clobetasone propionate under hydroactive dressings treatment of psoriatic plaques with the vitamin D_3 analogues Tacalcitol® (ointment 4μg/g) once daily[8] or with calcipotriol (cream 50μg/g) twice daily[13] interfered predominantly with epidermal proliferation resulting in a reduction of the number of basal keratinocytes in S- and G_2M phase by 34%. Vitamin D_3 analogues affected epidermal differentiation and inflammation less vigorously.

In the clinical part of the study the observation period after successful treatment with clobetasone propionate was 28 days. In this period 8 relapses (80%) occurred in the unoccluded sectors of the 10 cleared lesions. Occlusion reduced this percentage to 50% (5 relapses) and the combination of alcoholic solution and occlusion showed a further reduction to 3 relapses (30%). The 28-day observation period proved to be appropriate to show differences between different treatment modalities. The unoccluded sector of the clinical part was identically handled and therefore comparable to the lesion for the flow cytometric analysis. In the present study the relapse periods of the untreated sectors of the cleared lesion from the clinical part and the cleared lesion from the flow cytometric part showed no significant intraindividual variation, which implicates that within-patient comparison of topical treatment is justified.

The occlusion did not only reduce the number of relapses but had also significant influence on the clinical scores for erythema, induration and desquamation. As already mentioned above the combination of the alcoholic solution with hydroactive dressing further reduced the number of relapses compared to occlusion only. In particular the effect on the desquamation of the lesion was marked (Fig. 1c). No adverse events were encountered in association with the use of the alcoholic solution. Furthermore no influence on burning or itching of the lesion was reported. Therefore the alcoholic solution seems a safe vehicle for future research with active agents.

To study the early development of psoriatic lesions different approaches have been used in the past. Early lesions such as pinpoint papules, the margins of spreading plaques and relapsing psoriasis have been studied as models to investigate this process. Influx of polymorphonuclear leukocytes, monocytes and mast cells have been described as early events in developing lesions[1-3]. In spreading plaques and pinpoint lesions dermal or vascular changes have been described to precede epidermal changes[14-16]. One report, however, showed epidermal differentiation to be altered prior to vascular changes in the spreading psoriatic plaque[17].

The present evaluation of relapse after successful treatment with clobetasone propionate provides a new approach to study an early age of the psoriatic lesion the visibly unaffected skin of a healed lesion adjacent to newly developed pinpoint lesions The number of non-keratinocytes in the "unaffected skin at relapse" is not significantly different compared to the situation at clearance. In this "unaffected stage of relapsing skin" the percentage of cells in S- and G_2M phase in the basal cell population is intermediate between percentages at clearance and of lesional skin. However, the percentage of suprabasal keratinocytes in S- and G_2M phase in unaffected relapsing skin was already substantially increased with values comparable to lesional skin This model shows that the proliferative activity in the suprabasal compartment is an early aspect of epidermal proliferation in the pathogenesis of psoriasis.

In summary, we validated a model to analyse clearance and relapse characteristics of the psoriatic lesion, using clinical and flow cytometric parameters. It is attractive to speculate that this model will provide a new approach to compare different antipsoriatic maintenance therapies in vivo under standardized circumstances. Occlusion of psoriatic plaques with hydroactive dressings delays the process of relapse. Multiparameter flow cytometry provides quantitative information on these processes with respect to epidermal proliferation, differentiation and inflammation

ACKNOWLEDGEMENTS

The authors wish to thank Schering AG, Berlin, Germany for support of the present study

REFERENCES

1 Christophers E, Mrowietz U The inflammatory infiltrate in psoriasis Clinics in Dermatology 1995, 13 131-135

2 Chowaniec O, Jablonska S, Beutner EH, Proniewska M, Jarzabek-Chorzelska M, Rseza G Earliest clinical and histological changes in psoriasis Dermatologica 1981, 163 42-51

3 Schubert C, Christophers E Mast cells and macrophages in early relapsing psoriasis Arch Dermatol Res 1985, 277 352-358

4 Schalkwijk J, van Vlijmen-Willems IMJJ, Alkemade JAC, de Jongh GJ Immunohistochemical localization of SKALP/Elafin in psoriatic epidermis J Invest Dermatol 1993, 100 390-393

5 Gollnick H and Orfanos CE Retinoids In Mier, P D and van de Kerkhof, P C M eds Textbook of psoriasis Edinburgh Churchill Livingstone, 1995 252-267

6 van Erp PEJ, Rijzewijk JJ, Boezeman JBM, Leenders J, de Mare S, Schalkwijk J, et al Flow cytometric analysis of epidermal subpopulations from normal and psoriatic skin using monoclonal antibodies against intermediate filaments Am J Pathol 1989, 135 865-870

7 van Hooijdonk CAEM, Glade CP, van Erp PEJ TO-PRO-3 iodide, a novel HeNe laser-excitable DNA stain as an alternative for propidium iodide in multiparameter flow cytometry Cytometry 1994, 17 185-189

8 Glade CP, van Erp PEJ, van Hooijdonk CAEM, Elbers ME, van de Kerkhof PCM Topical treatment of psoriatic plaques with 1α,24 dihydroxyvitamin D_3 a multiparameter flow cytometrical analysis of epidermal growth, differentiation and inflammation Acta Derm Venereol (Stockh) 1995, 75 381-385

9 Glade CP, Seegers BAMPA, Meulen EFJ, van Hooijdonk CAEM, van Erp PEJ, van de Kerkhof PCM Multiparameter flow cytometric characterization of epidermal cell suspensions prepared from normal and hyperproliferative skin using an optimized thermolysin-trypsin protocol Arch Dermatol Res 1996, 288 203-210

10 Bauer FW and Boezeman JBM Flow cytometric methods in human skin with respect to cell cycle kinetics In Wright, N B and Camplejohn, R S eds Psoriasis Cell Proliferation Edinburgh Churchill Livingstone, 1983 104-116

11 Volden G Successful treatment of chronic skin diseases with clobetasol propionate and a hydrocolloid occlusive dressing Acta Derm Venereol (Stockh) 1992, 72 69-71

12 Goodwin P The effect of corticosteroids on cell turnover in the psoriatic patient Br J Dermatol 1976, 94 Suppl 12 95-100

13 Glade CP, van Erp PEJ and van de Kerkhof PCM Epidermal cell DNA content and intermediate filaments keratin 10 and vimentin after treatment of psoriatic plaques with calcipotriol cream once daily, twice daily and in combination with clobetasone 17-butyrate cream or betamethasone 17-valerate a comparative flow cytometric study Br J Dermatol 1996, 135 379-384

14 de Mare S, de Jong EMGJ, van Erp PEJ, van de Kerkhof PCM Markers for proliferation and keratinization in the margin of the active psoriatic lesion Br J Dermatol 1990, 122 469-475

15 de Jong EMGJ, Schalkwijk J, van de Kerkhof PCM Epidermal proliferation and differentiation, composition of the inflammatory infiltrate and the extracellular matrix in the margin of the spreading psoriatic lesion Eur J Dermatol 1991, 1 221-227

16 Goodfield M, Hull SM, Holland DB, Roberts SG, Wood EJ, Reid S, et al Investigations of the 'active' edge of plaque psoriasis vascular proliferation precedes changes in epidermal keratin Br J Dermatol 1994, 131 808-813

17 Parent D, Bernard BA, Desbas C, Heenen M, Darmon MY Spreading of psoriatic plaques alteration of epidermal differentiation precedes capillary leakiness and anomalies in vascular morphology J Invest Dermatol 1990, 95 333-340

Chapter 4

CLINICAL AND FLOW CYTOMETRIC EVALUATION OF NEW TOPICAL TREATMENT FOR PSORIASIS

4.1 Topical treatment of psoriatic plaques with 1α,24 dihydroxyvitamin D_3: a multiparameter flow cytometrical analysis of epidermal growth, differentiation and inflammation

Conrad P. Glade, Piet E.J. van Erp, Candida A.E.M. van Hooijdonk, Maria E. Elbers, Peter C.M. van de Kerkhof, Department of Dermatology, University Hospital Nijmegen, The Netherlands

Acta Derm Venereol (Stockh) 1995; 75: 381-385

SUMMARY

The clinical efficacy and tolerability of the vitamin D_3 analogues calcitriol, calcipotriol and 1α,24 dihydroxyvitamin D_3 in the treatment of psoriasis have been assessed in various clinical studies. In vitro and in vivo investigations have shown interference of these compounds with epidermal growth, keratinisation and inflammation. In this study we quantified the *in vivo* cell biological effects during treatment of psoriatic plaques with 1α,24 dihydroxyvitamin D_3. By using a flow cytometric triple labelling procedure we could discriminate different epidermal subpopulations, permitting precise assessment of epidermal cell cycle kinetics. Twenty patients with plaque-type psoriasis were treated in a double-blind placebo-controlled left-right comparative study with 1α,24 dihydroxyvitamin D_3 ointment (4μg/g applied once daily) for 8 weeks. Epidermal cell suspensions prepared from keratotome biopsies taken before and after treatment were stained with TO-PRO-3 iodide (a new DNA fluorochrome) and monoclonal antibodies against keratin 10 (as a marker for differentiation) and vimentin (as a marker for inflammation), simultaneously. The flow cytometric analyses showed a significant decrease of proliferating basal keratinocytes in verum-treated lesions, whereas such a decrease was not observed in placebo-treated lesions. The amount of keratin 10-positive keratinocytes increased and the presence of vimentin-positive cells decreased in cell suspensions derived from both verum- and placebo-treated lesions, but these effects were not significant. We conclude that multiparameter flow cytometry promises to be an adequate approach to assess the interference of antipsoriatic treatments with cutaneous inflammation, epidermal proliferation and keratinisation. Topical 1α,24 dihydroxyvitamin D_3 seems to exert its *in vivo* antipsoriatic effect mainly through an inhibition of epidermal growth.

INTRODUCTION

In 1985 Morimoto & Kumahara described a patient with senile osteoporosis whose psoriasis cured after oral administration of 1α hydroxyvitamin D_3, a prodrug of calcitriol[1]. Their observation caused a renewed interest in the class of vitamin D_3 analogues as an antipsoriatic principle[2,3]. During the last decade the clinical efficacy of 1α,25 dihydroxyvitamin D_3 (calcitriol)[4-8] and several of its derivatives, including 1α monohydroxyvitamin D_3[4], calcipotriol[9-12] and 1α,24 dihydroxy-vitamin D_3 (1α,24 $(OH)_2D_3$) (Tacalcitol)[13-15] has been documented. Calcipotriol (50μg/g), 1α,24 $(OH)_2$ D_3 (2-4μg/g) and calcitriol (3-15μg/g) can be applied topically. Calcipotriol and 1α,24 $(OH)_2$ D_3 exert a decreased calcitropic effect compared to calcitriol[15,16]. Furthermore, neither hypercalcemia nor hypercalciurea were observed in patients who received a daily total dose of 80 μg 1α,24 $(OH)_2$ D_3 for 7 days[17]. Calcitriol induced irritation of the skin at concentrations of 15μg/g in 6.3% of the patients, whereas concentrations of 3μg/g caused irritation in 3.4% of patients[7,8]. Irritation of the skin, especially of the face, was seen in 4.3-19,5% of patients treated with calcipotriol[11,18]. Remarkably, with respect to 1α,24 $(OH)_2D_3$, so far, only a slight irritation has been reported in less than 1% of patients[15]. Therefore, 1α,24 $(OH)_2D_3$ has expanded the horizon of vitamin D_3 treatment of psoriasis, as facial and flexural lesions can be treated with this analogue without significant irritation.

In vitro data on mouse and human keratinocytes and in vivo data on guinea pigs, but also studies in psoriatic patients have shown the capability of 1α,24 $(OH)_2D_3$ to suppress DNA synthesis and to induce epidermal differentiation[16,19-22]. These findings have been confirmed in psoriasis *in vivo* by a previous immunohistochemical study from our department, showing a substantial inhibitory effect of 1α,24 $(OH)_2D_3$ with respect to epidermal proliferation and a significant modulation of cutaneous inflammation and keratinisation[23]. Histological scoring of stained sections, however, is semi-quantitative. A more objective and quantitative method is required for an accurate and reliable assessment of the effect of antipsoriatic therapy.

Multiparameter flow cytometry allows a simultaneous, quantitative, statistically accurate analysis of different cell parameters and is a useful tool for studies on skin pathology[24,25]. Triple labelling combining double immunophenotyping and assessment of DNA content is impeded by a considerable spectral overlap[26-28]. Only to a limited extent could these difficulties be overcome[29,30]. Recently, by using the new DNA stain TO-PRO-3 iodide (TP3), we were able to design a procedure in which DNA content and expression of two intermediate filament proteins were measured simultaneously in

single cell suspensions from epidermis[31]. Simultaneous discrimination and quantification of three different epidermal subpopulations, i.e. non-keratinocytes, differentiated and basal keratinocytes, became possible, allowing a precise quantification of the proliferative activity of the basal compartment.

The aim of the present study was to quantify flow cytometrically in terms of epidermal proliferation, differentiation and inflammation, the response of the psoriatic lesion to a therapeutical concentration of 1α,24 $(OH)_2D_3$ (4μg/g Tacalcitol ointment applied once daily).

MATERIALS AND METHODS

Patients and skin samples

Twenty patients (11 females and 9 males; age range 22-66 years, mean age 43.5 years) with symmetrically distributed chronic plaque psoriasis participated in the investigation, after informed consent had been given. The mean duration of psoriasis was 20.2 years. The study design was a placebo-controlled double-blind left-right comparison. 1α,24 $(OH)_2D_3$ (Tacalcitol, Hermal AG, Hamburg, Germany) was manufactured in an ointment base consisting of paraffinum subliquidum, diisopropyladipat and vaseline album. The verum comprised 4μg/g 1α,24 $(OH)_2D_3$ ointment. The placebo contained the ointment base only. In the previous 2 months, the patients had not received systemic therapy, and in the previous 4 weeks no topical treatment had been applied. After an initial wash-out phase of 2 weeks patients were treated for 8 weeks with 1α,24 $(OH)_2D_3$ on one body halve and placebo on the other body halve. The maximum dose of 1α,24 $(OH)_2D_3$ was 40 μg per day. Before and during treatment clinical improvement was assessed using the Psoriasis Area and Severity Index, including scoring for erythema, induration and scaling using a five point scale. Blood investigations were carried out every 2 weeks.

In each patient two symmetrically localised lesions were selected for the cell biological assessment. Before treatment keratotome biopsies (0,4 mm thick and ± 1 cm^2) using a small dermatotome (Coriotome 6B333, Aesculap AG, Tuttlingen, Germany) were taken from these lesions. After 8 weeks of treatment biopsies were taken from the same areas. In this way, from each patient four biopsies (two 1α,24 $(OH)_2D_3$-treated and two placebo-treated) were obtained. Before initiation of the study Medical Ethical Committee approval had been obtained.

Preparation of cell suspension

Epidermal cell suspensions were prepared using a modification of a trypsinisation procedure previously described by Gommans et al.[32]. In brief, the biopsies were washed in phosphate-buffered saline (PBS) and floated with dermal side downwards on a solution containing 0.025% w/v trypsin (Sigma, St. Louis, USA) and 0.3% w/v dithioerythritol (Sigma, St. Louis, USA) in PBS for 30 min at 37° C. Then, in a solution containing 10% v/v heat inactivated newborn calf serum (HINCS, Life Technologies Ltd., Paisley, UK) in PBS, the dermis was peeled off with a fine forceps. After the basal cells had been removed by waving in the solution, the dermis was discarded. After gentle agitation ("Vortex"mixer) of the remaining epidermis for 1 min, the transparent horny layer was removed. The epidermal cells obtained in this way were centrifuged , resuspended in 2-3 ml ice-cold ethanol (70% v/v) and stored at - 20°C until use.

Staining procedure

A triple labelling was performed. DNA content was measured using the new DNA fluorochrome TO-PRO-3 iodide (TP3, Molecular Probes, Eugene, USA). To assess epidermal keratinisation, the IgG_1-type monoclonal antibody RKSE60 (gift from Prof. F.C. Ramaekers, Department of Molecular Biology, University of Maastricht, The Netherlands) was used. RKSE60 is directed against keratin 10, an intermediate filament-type protein of 56.5 kD that is exclusively expressed in suprabasal, i.e. differentiating keratinocytes in normal and psoriatic skin[33]. To assess epidermal inflammation, the IgG_{2a}-type monoclonal antibody Vim3B4 (Novocastra Laboratories ltd., Newcastle upon Tyne, UK) was used. Vim3B4 is directed against vimentin, the intermediate filament-type protein that is expressed in cells of mesenchymal origin[34,35]. In the epidermis Vim3B4 stains non-keratinocytes, i.e. dendritic cells and (especially in psoriatic skin) inflammatory infiltrate cells. The second step of the indirect immunofluorescent staining was performed with monoclonal goat antibodies against mouse IgG_1 and IgG_{2a}, conjugated to phycoerythrin (PE) and fluorescein-isothiocyanate (FITC), respectively (Southern Biotechnology Associates, Birmingham, USA).

Aliquots (750µl) of the cell suspensions, which contained $1\text{-}2 \times 10^5$ cells, were washed in PBS, filtered to remove clumps and horny material, and resuspended in 500µl of a solution with Vim3B4 diluted 1:50 and RKSE60 diluted 1:15 in PBS. After incubation for 30 min at room temperature in the dark, the cells were washed in PBS containing

1% HINCS, resuspended and incubated for 15 min at 5°C in a solution of 500µl PBS, containing 2µl goat-anti-mouse-PE, 10µl goat-anti-mouse-FITC, 10µl normal goat serum and 5µl HINCS. After a third washing step DNA staining was performed by addition of 300µl TP3 (1µM in PBS) and 50µl RNase (1mg/ml in PBS) (Sigma, St. Louis, USA).

Flow cytometric analysis

From each sample 5,000 gated cells were measured and analysed using an EPICS® Elite flow cytometer (Coulter, Luton, UK), equipped with a dual laser system. Both FITC and PE were excited with an air-cooled argon ion laser (15mW, 488nm). TP3 was excited with a HeNe laser (10mW, 633nm). After determination of the electronic compensation for spectral overlap in earlier experiments, which was minimal in the case of TP3, fluorescence was measured using bandpass filters at 525nm (green, FITC), 575nm (orange, PE) and 675nm (red, TP3). The ratio area/peak of the red signal (DNA) was used to discriminate between doublets of diploid cells and real tetraploid cells[25]. After setting appropriate gates with the Elite software, percentages of vimentin- and keratin 10-positive cells were calculated. With the aid of Multicycle™ software (Phoenix, Flow Systems, San Diego, USA) the percentage of basal keratinocytes in SG_2M phase of the cell cycle was calculated from DNA histograms.

RESULTS

Clinical response

Thirteen patients showed a moderate to marked improvement of their psoriatic lesions. One patient, who also showed a marked improvement, was withdrawn from the study after 6 weeks because of an exacerbation of psoriatic arthritis, requiring additional medication. The mean PASI score showed a significant reduction after 8 weeks of treatment for both 1α,24 $(OH)_2D_3$- and placebo-treated lesions. 1α,24 $(OH)_2D_3$-treated lesions showed a mean reduction of the PASI score of 48%. The pretreatment PASI score was 5.3 ± 0.4 (mean ± SEM) and the posttreatment PASI score was 2.8 ± 0.4 ($p \leq 0.0001$, Wilcoxon test for matched pairs). The mean PASI score in placebo-treated lesions decreased 28% (pretreatment 5.4 ± 0.4 and posttreatment 3.9 ± 0.4, $p \leq 0.0001$). Comparison of posttreatment PASI scores between the 1α,24 $(OH)_2D_3$- and placebo-treated lesions showed a significant difference in favour of the 1α,24 $(OH)_2D_3$ treated lesions ($p \leq 0.01$).

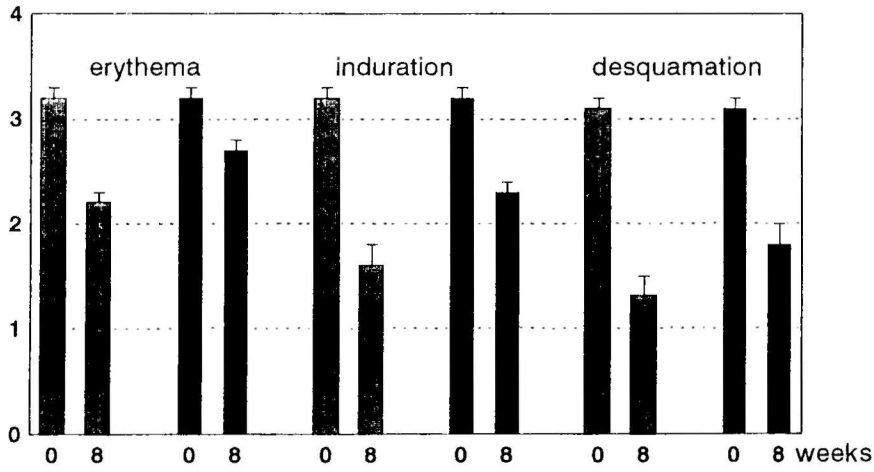


Figure 1. Clinical scores before and after 8 weeks treatment with 1α,24 dihydroxyvitamin D_3 (4μg/g) ointment (shaded bars) and ointment base only (black bars).

As shown in Fig. 1 the clinical severity scores for erythema, induration and desquamation of the biopsied lesions all showed a significant reduction, which was more substantial for 1α,24 $(OH)_2D_3$-treated lesions. The mean score for erythema in 1α,24 $(OH)_2D_3$-treated lesions decreased from 3.2 ± 0.1 to 2.2 ± 0.1 ($p \leq 0.0004$), and in placebo-treated lesions from 3.2 ± 0.1 to 2.7 ± 0.1 ($p \leq 0.008$). The mean score for induration showed a reduction from 3.2 ± 0.1 to 1.6 ± 0.2 ($p \leq 0.0003$) in 1α,24 $(OH)_2D_3$-treated, and a reduction from 3.2 ± 0.1 to 2.3 ± 0.1 ($p \leq 0.0007$) in placebo-treated lesions. With respect to desquamation the mean score in 1α,24 $(OH)_2D_3$-treated lesions decreased from 3.1 ± 0.1 to 1.3 ± 0.2 ($p \leq 0.0003$), and in placebo-treated lesions from 3.1 ± 0.1 to 1.8 ± 0.2 ($p \leq 0.0007$). Comparison of posttreatment clinical scores revealed a significant difference. The reductions of erythema, induration and scaling were more substantial at the 1α,24 $(OH)_2D_3$-treated sites compared to placebo-treated sites at the levels $p \leq 0.005$, $p \leq 0.008$ and $p \leq 0.02$, respectively.

The blood investigations, in particular calcium metabolism, did not show any significant aberration. No side-effects were seen during the study period. In none of the patients was any skin irritation noticed.

Flow cytometric results

Typical DNA histograms representative for untreated and treated psoriatic plaques are shown in Fig. 2. Fluorescence of TP3 (red signal) was measured of cells which were vimentin-negative (green signal) and keratin 10-negative (orange signal). By distinction of subpopulations in this way, DNA content of the basal keratinocytes could be assessed. In this example, in the psoriatic lesion before treatment, we found a percentage of cells (i.e. of basal keratinocytes) in S- and G_2M phase of 17.5%. Eight weeks later this percentage was reduced to 12.8%. Data analysis was restricted to patients from whom all four epidermal cell suspensions contained a cell amount of at least 30%. In this way, paired data from 12 patients were obtained.

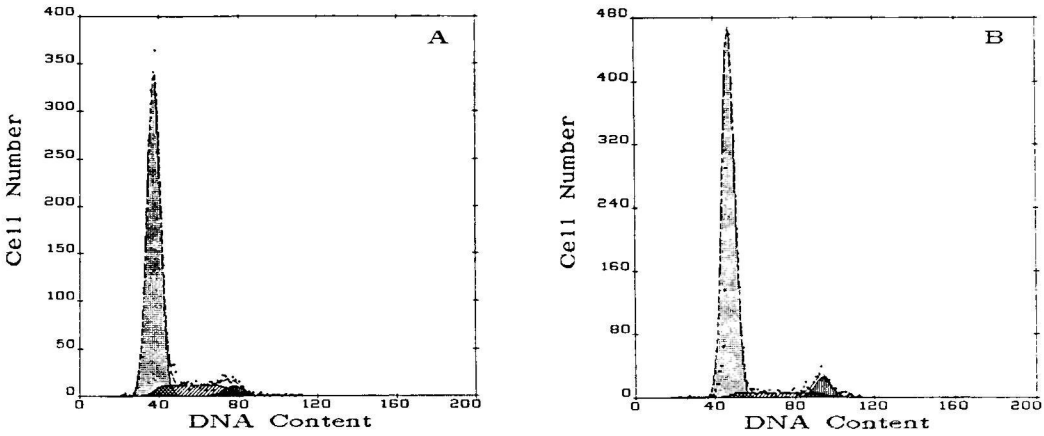


Figure 2. DNA histograms of epidermal cell suspensions prepared from keratotome biopsies taken from psoriatic plaques (a) before and (b) after 8 weeks treatment with 1α,24 dihydroxyvitamin D_3 (4μg/g) ointment.

For placebo-treated lesions the average percentage of cells in S- and G_2M phase was 17.8 ± 1.8% before treatment. After treatment a tendency of a reduction was seen to 15.1 ± 1.2% ($p \leq 0.35$, Wilcoxon test for matched pairs). In 1α,24 $(OH)_2D_3$-treated lesions the mean pretreatment percentage of cells in S- and G_2M phase was 20.0 ±

1 9% After 8 weeks of treatment a more substantial and significant decrease to 13 2 ± 1 1 ($p \leq 0$ 01) was seen The mean pretreatment percentage of vimentin-positive cells proved to be 15 1 ± 2 4% for 1α,24 $(OH)_2D_3$-treated lesions and 16 2 ± 2 5% for placebo-treated lesions After 8 weeks a reduction was seen for both sides to 12 9 ± 1 4% ($p \leq 0$ 66) and 13 2 ± 1 8% ($p \leq 0$ 14), respectively In neither case was this a significant reduction With respect to keratin 10 expression, the mean pretreatment percentage of positive cells was 37 7 ± 3 7% for 1α,24 $(OH)_2D_3$-treated lesions and 35 2 ± 3 0% for placebo-treated lesions The mean posttreatment percentages showed an increase to 43 4 ± 3 5 % ($p \leq 0$ 43) and 47 9 ± 3 7% ($p \leq 0$ 02), respectively

DISCUSSION

In the present investigation we assessed the effect of topically applied 1α,24 $(OH)_2D_3$ ointment (4μg/g) on proliferation, differentiation and inflammation in the psoriatic plaque using multiparameter flow cytometry In 1α,24 $(OH)_2D_3$-treated lesions we found a statistically significant reduction of the percentage of basal cells in S- and G_2M phase (as marker for proliferation) from 20 0% to 13 2% This reduction was less and not significant in placebo-treated lesions The amount of keratin 10-positive keratinocytes (as marker for differentiation) increased after 8 weeks The decrease of vimentin-positive cells (as marker for inflammation) was in the same range for both 1α,24 $(OH)_2D_3$- and placebo-treated lesions

The clinical efficacy of 1α,24 $(OH)_2D_3$ D_3 ointment (4μg/g once daily) as a topical antipsoriatic agent is confirmed in this study The mean reduction of the PASI score, as a result of 8 weeks' treatment with 1α,24 $(OH)_2D_3$ proved to be 48% (present study) This reduction is in line with our previous report showing 49% reduction of PASI scores by 1α,24 $(OH)_2D_3$[23] Following a 6 weeks' treatment with calcipotriol ointment (50μg/g) twice daily and betamethasone valerate ointment twice daily, the mean reduction of PASI scores were 68 8% and 61 4%, respectively[11] So far, however, no comparative studies of calcipotriol and 1α,24 dihydroxyvitamin D_3 are available The absence of skin irritation and hypercalcemia in all patients validates earlier statements on 1α,24 $(OH)_2D_3$[15]

In vitro, 1α,24 $(OH)_2D_3$ has been shown to inhibit proliferation of keratinocytes[16 19 20] In the immunohistochemical study 1α,24 $(OH)_2D_3$ proved to inhibit recruitment of cycling epidermal cells profoundly[23] In the present study a reduction of the percentage basal cells in S- and G_2M phase of 34% was reached In a previous flow cytometrical

study from our department on the in vivo effect of calcipotriol, a 31% reduction of the percentage of cells in S- and G_2M phase from 11.8% (before treatment) to 8.2% (after 6 weeks' treatment) was shown[36]. An important methodological difference between the two studies is that the percentage of cells in S- and G_2M phase was limited to the basal keratinocytes only in the present study, in contrast to all epidermal cells in the previous study. By using a triple labelling method, non-keratinocytes and differentiated keratinocytes were excluded, which permits a precise and specific assessment of epidermal cell cycle kinetics.

The absence of a significant change in the relative number of keratin 10-positive cells after treatment with 1α,24 $(OH)_2D_3$ could suggest that basal and suprabasal compartments maintain the same relative size before and after treatment. Previous flow cytometric studies, however, have demonstrated the relative numbers of keratin 10-positive cells in untreated psoriatic plaques (46.6%) to be reduced compared to normal skin (57.2%)[24]. This means that, though a normalization of keratin 10 expression can be produced by calcipotriol[37], 1α,24 $(OH)_2D_3$ does not seem to influence this expression significantly.

In vitro studies have demonstrated a direct effect of 1α,24 $(OH)_2D_3$ on inflammatory cells. A dose-dependent induction of differentiation and suppression of antibody response to T cell dependent antigen by 1α,24 $(OH)_2D_3$ has been shown[38]. The functional properties of neutrophils *in vitro* were not modulated by 1α,24 $(OH)_2D_3$[15,21]. In a previous *in vivo* study, an 8-week treatment period of 1α,24 $(OH)_2D_3$ had resulted in a significant reduction of the number of T cells, monocytes and neutrophils in the inflammatory infiltrate of the psoriatic lesion[23]. However, in the present study only a tendency of a reduction of the relative number of vimentin-positive cells, i.e. all mesenchymal cells, including the infiltrate cells, was observed. This discrepancy might be explained by the fact that the total number of cells present per surface area (mesenchymal cells and keratinocytes) has decreased following treatment[35]. This implicates that the decrease in the absolute number of vimentin-positive cells was in the same range as the decrease of all epidermal cells obtained from the biopsy. Further studies will unravel the relative changes in populations of infiltrate cells during treatment.

The present study lends support for the hypothesis that topical 1α,24 $(OH)_2D_3$ exhibits its antipsoriatic potential mainly through an inhibiting effect on epidermal growth. The influence on epidermal inflammation and keratinisation is limited. Three-color flow cytometry seems to be an attractive tool to make an objective assessment of the effect

of antipsoriatic therapy on different cell parameters. Validation of the usefulness of this single measurement for proliferation, differentiation and inflammation can be reached by evaluation of other therapy modalities.

REFERENCES

1. Morimoto S, Kumahara Y. A patient with psoriasis cured by 1a-hydroxyvitamin D_3. Med J Osaka Univ 1985; 35: 51-54.
2. Kragballe K. Vitamin D3 and skin diseases. Arch Dermatol Res 1992; 284 Suppl 1: S30-S36.
3. van de Kerkhof PCM. Vitamin D3 und seine Analoge in der Dermatologie. Z Hautkr 1994; 69: 219-226.
4. Morimoto S, Yoshikawa K, Kozuka T, Kitano Y, Imanaka S, Fukuo K, et al. An open study of vitamin D3 treatment in psoriasis vulgaris. Br J Dermatol 1986; 115: 421-429.
5. Smith EL, Pincus SH, Donovan L, Holick MF. A novel approach for the evaluation and treatment of psoriasis. Oral or topical use of 1,25-dihydroxyvitamin D3 can be a safe and effective therapy for psoriasis. J Am Acad Dermatol 1988; 19: 516-528
6. van de Kerkhof PCM, van Bokhoven M, Zultak M, Czarnetzki BM. A double-blind study of topical 1 alpha,25-dihydroxyvitamin D3 in psoriasis. Br J Dermatol 1989; 120: 661-664.
7. Langner A, Verjans H, Stapor V, Mol M, Fraczykowska M Topical calcitriol in the treatment of chronic plaque psoriasis: a double-blind study. Br J Dermatol 1993, 128: 566-571.
8. Langner A, Verjans H, Stapor V, Mol M, Fraczykowska M. 1a,25-Dihydroxyvitamin D_3 (calcitriol) ointment in psoriasis. J Dermatol Treatment 1992; 3: 177-180.
9. Kragballe K, Beck HI, Sogaard H. Improvement of psoriasis by a topical vitamin D3 analogue (MC 903) in a double-blind study. Br J Dermatol 1988, 119: 223-230
10. Staberg B, Roed Petersen J, Menne T. Efficacy of topical treatment in psoriasis with MC903, a new vitamin D analogue. Acta Derm Venereol (Stockh) 1989; 69: 147-150
11. Kragballe K, Gjertsen BT, De Hoop D, Karlsmark T, van de Kerkhof PCM, Larkö O, et al. Double-blind, right/left comparison of calcipotriol and betamethasone valerate in treatment of psoriasis vulgaris. Lancet 1991; 337: 193-196.
12. Kragballe K. Treatment of psoriasis by the topical application of the novel cholecalciferol analogue calcipotriol (MC 903). Arch Dermatol 1989; 125: 1647-1652.
13. Kato T, Rokugo M, Terui T, Tagami H. Successful treatment of psoriasis with topical application of active vitamin D3 analogue, 1 alpha,24-dihydroxycholecalciferol. Br J Dermatol 1986; 115: 431-433.
14. TV-02 Ointment Research Group. A placebo-controlled double-blind, right/left comparison study on the efficacy of TV-02 ointment for the treatment of psoriasis. Nishi Nihon J Dermatol 1991; 53: 1252-1261.
15. Nishimura M, Hori Y, Nishiyama S, Nakamizo Y. Topical 1a,24 (R)-dihydroxyvitamin D_3 for the treatment of psoriasis. Review of the literature. Eur J Dermatol 1993; 3: 255-261.

16 Matsunaga T, Yamamoto M, Mimura H, Ohta T, Kiyoki M, Ohba T, et al 1,24(R)-dihydroxyvitamin D3, a novel active form of vitamin D3 with high activity for inducing epidermal differentiation but decreased hypercalcemic activity J Dermatol 1990, 17 135-142

17 TV-02 Ointment Research Group Assessment of the safety of TV-02 ointment for patients with psoriasis Nishi Nihon J Dermatol 1989, 51 317-324

18 Cunliffe WJ, Berth-Jones J, Claudy A, Fairiss G, Goldin D, Gratton D, et al Comparative study of calcipotriol (MC 903) ointment and betamethasone 17-valerate ointment in patients with psoriasis vulgaris J Am Acad Dermatol 1992, 26 736-743

19 Kobayashi T, Okumura H, Azuma Y, Kiyoki M, Matsumoto K, Hashimoto K, et al 1 alpha,24R-dihydroxyvitamin D3 has an ability comparable to that of 1 alpha,25-dihydroxyvitamin D3 to induce keratinocyte differentiation J Dermatol 1990, 17 707-709

20 Matsumoto K, Hashimoto K, Kiyoki M, Yamamoto M, Yoshikawa K Effect of 1,24R-dihydroxyvitamin D3 on the growth of human keratinocytes J Dermatol 1990, 17 97-103

21 Kato T, Terui T, Tagami H Topically active vitamin D3 analogue, 1 alpha,24-dihydroxy-cholecalciferol, has an anti-proliferative effect on the epidermis of guinea pig skin Br J Dermatol 1987, 117 528-530

22 Kawara S, Hatta N, Hirone K Effect of externally applied 1a, 24 (R)-$(OH)_2$ D_3 on the kinetics of epidermal keratinocytes in the psoriatic focus Japanese Journal of Dermatology 1989, 13 99

23 Gerritsen MJP, Boezeman JBM, van Vlijmen-Willems IMJJ, van de Kerkhof PCM The effect of Tacalcitol (1,24 $(OH)_2$ D_3) on cutaneous inflammation, epidermal proliferation and keratinization in psoriasis, a placebo-controlled, double-blind study Br J Dermatol, 1994, 131 57-63

24 van Erp PEJ, Rijzewijk JJ, Boezeman JB, Leenders J, de Mare S, Schalkwijk J, et al Flow cytometric analysis of epidermal subpopulations from normal and psoriatic skin using monoclonal antibodies against intermediate filaments Am J Pathol 1989, 135 865-870

25 Bauer FW and Boezeman JBM Flow cytometric methods in human skin with respect to cell cycle kinetics In Wright NB, Camplejohn RS, eds Psoriasis cell proliferation Edinburgh Churchill Livingstone, 1983 104-116

26 Rabinovitch PS, Torres RM, Engel D Simultaneous cell cycle analysis and two-color surface immunofluorescence using 7-amino-actinomycin D and single laser excitation applications to study of cell activation and the cell cycle of murine Ly-1 B cells J Immunol 1986, 136 2769-2775

27 Schmid I, Krall WJ, Uittenbogaart CH, Braun J, Giorgi JV Dead cell discrimination with 7-amino-actinomycin D in combination with dual color immunofluorescence in single laser flow cytometry Cytometry 1992, 13 204-208

28 Toba K, Winston EF, Bray RA Improved staining method for the simultaneous flow cytofluorometric analysis of DNA content, S-phase fraction, and surface phenotype using single laser instrumentation Cytometry 1992, 13 60-67

29 Begg AC, Hofland I Cell kinetic analysis of mixed populations using three-color fluorescence flow cytometry Cytometry 1991, 12 445-454

30 Corver WE, Cornelisse CJ, Fleuren GJ Simultaneous measurement of two cellular antigens and DNA using fluorescein-isothiocyanate, R-phycoerythrin, and propidium iodide on a standard FACScan Cytometry 1994, 15 117-128

31 van Hooijdonk CAEM, Glade CP, Van Erp PEJ TO-PRO-3 iodide, a novel HeNe laser-excitable DNA stain as an alternative for propidium iodide in multiparameter flow cytometry Cytometry 1994, 17 185-189

32 Gommans JM, Bergers M, van Erp PEJ, van den Hurk JJ, Mier PD, Roelfzema H Studies on the plasma membrane of normal and psoriatic keratinocytes I Preparation of material and morphological characterization Br J Dermatol 1979, 101 407-412

33 Ramaekers FC, Puts JJ, Moesker O, Kant A, Huysmans A, Haag D, et al Antibodies to intermediate filament proteins in the immunohistochemical identification of human tumours an overview Histochem J 1983, 15 691-713

34 de Waal RM, Semeijn JT, Cornelissen MH, Ramaekers FC Epidermal Langerhans cells contain intermediate-sized filaments of the vimentin type an immunocytologic study J Invest Dermatol 1984, 82 602-604

35 Bauer FW, Boezeman JB, van Engelen L, de Grood RM, Ramaekers FC Monoclonal antibodies for epidermal population analysis J Invest Dermatol 1986, 87 72-75

36 de Mare S, De Jong EGJM, van de Kerkhof PCM DNA content and K_s8 12 binding of the psoriatic plaque lesion during treatment with the vitamin D_3 analogue MC903 and betamethasone Br J Dermatol 1990, 123 291-295

37 Berth-Jones J, Fletcher A, Hutchinson PE Epidermal cytokeratin and immunocyte responses during treatment of psoriasis with calcipotriol and betamethasone valerate Br J Dermatol 1992, 126 356-361

38 Komoriya K, Nagata I, Tsuchimoto M, Kunisawa K, Takeshita T Naruchi T 1,25-Dihydroxyvitamin D3 and 1,24-dihydroxyvitamin D3 suppress in vitro antibody response to T cell-dependent antigen Biochem Biophys Res Commun 1985, 127 753-758

4.2 Epidermal cell DNA content and intermediate filaments keratin 10 and vimentin after treatment of psoriasis with calcipotriol cream once daily, twice daily and in combination with clobetasone 17-butyrate cream or betamethasone 17-valerate cream: a comparative flow cytometric study

Conrad P Glade, Piet E.J van Erp, Peter C M. van de Kerkhof
Department of Dermatology, University Hospital Nijmegen, The Netherlands
Br J Dermatol 1996; 135: 379-384

SUMMARY

Calcipotriol and corticosteroids, two therapy modalities frequently prescribed in the treatment of psoriasis, are often used in combination The aim of the present study was to determine whether the cell biological response pattern of concurrent use of calcipotriol and corticosteroids is different from calcipotriol monotherapy. Forty patients with a chronic plaque psoriasis were divided at random in four parallel groups and treated for 8 weeks with (1) calcipotriol cream (50 μg/g once daily); (2) calcipotriol cream twice daily, (3) calcipotriol and clobetasone 17-butyrate (0 5 mg/g) creams, and 4) calcipotriol and betamethasone 17-valerate (1 mg/g) creams. Before and after treatment keratotome biopsies were taken and single cell suspensions prepared for flow cytometric analysis Flow cytometric multiparameter quantification of markers for proliferation (TO-PRO-3), differentiation (antikeratin 10) and inflammation (antivimentin) was used to evaluate all four therapy modalities.

A statistically significant decrease of the percentage of basal cells in S- and G_2M-phase (proliferation) was obtained with all therapy modalities, except for calcipotriol monotherapy applied once daily A significant reduction of the number of vimentin-positive cells (non-keratinocytes) was observed following combined treatment with calcipotriol and clobetasone butyrate. In contrast, monotherapy with calcipotriol had virtually no effect on the number of vimentin-positive cells.

It can be concluded that: (i) calcipotriol monotherapy, applied once daily was less antiproliferative compared with twice daily applications of calcipotriol or the combined treatment with corticosteroids and that (ii) the combination of calcipotriol

and corticosteroids proved to have a marked effect on the percentage of non-keratinocytes, in contrast to the modest effect of calcipotriol.

INTRODUCTION

Vitamin D_3 analogues interfere with various characteristics of the psoriatic plaque: epidermal hyperproliferation, impaired differentiation and cutaneous inflammation. *In vitro* studies demonstrated that calcipotriol inhibits proliferation and stimulates differentiation in cultured human keratinocytes. Several inflammatory processes are modulated by calcipotriol.[1-5] *In vivo*, topical application of calcipotriol to psoriatic plaques resulted in decreased epidermal DNA synthesis and a shift of the expression pattern of epidermal cytokeratins towards normalization.[6-9] Furthermore calcipotriol has been reported to influence different cytokines[10] and to change the numbers of several immunocytes (polymorphonuclear leukocytes, CD1a positive cells, T lymphocytes, monocytes) in psoriatic plaques.[8,9,11,12] Interference with epidermal proliferation has been reported to be the most conspicuous effect of calcipotriol treatment, whereas interference with inflammation is less marked.[9] Using absolute counts, however, a significant reduction of infiltrate cells in the epidermis has been claimed.[8] In this respect it is of relevance that the combination of topical treatment with calcipotriol and low-dose systemic cyclosporin (2 mg/kg/day) proved to be a highly effective combination, indicating that the immunomodulating effect of cyclosporin might compensate for the relatively low immunosuppressive capacity of calcipotriol *in vivo*.[13]

Double-blind, placebo-controlled studies showed that effective and safe antipsoriatic therapy can be obtained with calcipotriol in a concentration of 50 μg/g.[14,15] In a right-left comparison calcipotriol induced a significantly larger reduction in PASI score compared with 0·1% betamethasone 17-valerate ointment.[16] In a parallel group study no significant difference in the reduction of PASI score was demonstrated between calcipotriol and betamethasone, but patients' efficacy assessment was significantly in favour of calcipotriol.[17] Calcipotriol ointment was also more effective and better accepted than short-contact dithranol therapy.[18] However, an important limitation of calcipotriol monotherapy twice daily is the occurrence of irritative dermatitis in 10-20% of patients.[19] Once daily application of calcipotriol might decrease side-effects while maintaining clinical efficacy. Also concurrent use of topical calcipotriol and topical corticosteroids might reduce the frequency of irritative dermatitis while

maintaining or even improving the clinical result. Recently, a large multicentre study was initiated to establish the clinical efficacy and tolerability of a once-daily schedule of calcipotriol treatment and a combination of calcipotriol with a low and a medium strength corticosteroid. The results of this study (Leo Pharmaceutical Products, Denmark, data on file) will be published as a full report.

The aim of the present study was to analyse the response pattern of psoriatic skin with respect to epidermal proliferation (percentage basal cells in S- and G_2M-phase), epidermal differentiation (percentage cells expressing the differentiation marker keratin 10) and cutaneous inflammation (relative number of vimentin-positive cells) to various treatment schedules: (1) calcipotriol cream once daily; (2) calcipotriol cream twice daily; (3) calcipotriol cream in combination with clobetasone 17-butyrate cream (0·5 mg/g); or (4) calcipotriol cream in combination with betamethasone 17-valerate cream (1 mg/g). These response patterns were evaluated using a recently developed flow cytometric multiparameter technique with simultaneous quantification of DNA content and two intermediate filament proteins in epidermal single cell suspensions.[20,21] Single cell suspensions were prepared from skin samples of 40 patients who participated in the above-mentioned comparative double-blind parallel group multicentre study at the Department of Dermatology, Nijmegen.

MATERIALS AND METHODS

Patients and biopsies

Forty patients with stable chronic plaque psoriasis participated in the study after informed consent. No systemic treatment had been used for at least 6 weeks. Excluded were patients who were pregnant, breastfeeding or expected to become pregnant. After a wash-out period of 2 weeks, in which only an emollient (Danatekt®) was permitted, patients were at random assigned to one of four therapy groups. These consisted of 8 weeks treatment with a morning application of calcipotriol cream 50 μg/g and an evening application of either (1) vehicle of calcipotriol cream, (2) calcipotriol cream 50 μg/g, (3) clobetasone butyrate cream 0·5 mg/g or (4) betamethasone valerate cream 1 mg/g on psoriatic lesions of extremities and trunk. Assessment of clinical scores for erythema, induration and desquamation (five-point scale) and of the area of the psoriatic lesions was performed every 2 weeks. Patients were withdrawn from the study before 8 weeks treatment if all lesions had cleared. Before and after treatment in

total two keratotome biopsies (thickness 0 4 mm, 2 cm^2) were taken from the same test lesion for flow cytometric analysis

Cell isolation procedure

Epidermal single cell suspensions were prepared as described before.[21] Briefly, the keratotome biopsies were incubated in phosphate-buffered saline (PBS) containing 0·25 mg/ml trypsin (Sigma, St Louis, MO, U.S A.) and 3 0 mg/ml dithioerythritol (Sigma) for 30 min at 37°C. Then, in PBS containing 10% heat-inactivated newborn calf serum (HINCS, Life Technologies Ltd., Paisley, U K.) the dermis was separated from the epidermis with a fine forceps. The remaining epidermis was gently mixed on a vortex to loosen the keratinocytes, resulting in a single cell suspension. After discarding the horny layer, the suspension was centrifuged, the supernatant removed and the cells fixed in 70% ice-cold ethanol. The cell suspension was stored at -20°C until staining and flow cytometric analysis.

Staining procedure

Triple labelling was performed, using a DNA fluorochrome combined with two antibodies against intermediate filaments. The procedure has been described by us in detail before [21] To assess proliferation, the DNA fluorochrome TO-PRO-3 iodide (TP3, Molecular Probes, Eugene, OR, U.S.A.) was used [20] TP3 intercalates with double-stranded DNA and permits measurement of the proliferative activity of cells by quantification of the percentage of cells in S- and G_2M-phase. As TP3 also binds to RNA to some extent, it was used in combination with RNase To study inflammation Vim3B4 (Novocastra Laboratories Ltd , Newcastle upon Tyne, U K) was used This IgG2a-type mouse monoclonal antibody stains vimentin, an intermediate filament-type protein which occurs in mesenchymal cells.[22] In the present study, PMNs, lymphocytes, monocytes, macrophages, melanocytes and Langerhans cells were stained by Vim3B4 The IgG1-type mouse monoclonal antibody RKSE60 (Department of Molecular Biology, University of Maastricht, The Netherlands) was used as differentiation marker. RKSE60 is directed against keratin 10, an intermediate filament-type protein that is expressed in differentiating keratinocytes [23 24] Three-colour fluorescence was obtained with the fluorochromes fluorescein-isothiocyanate (FITC) and phycoerythrin (PE) which were conjugated to monoclonal goat antibodies against mouse IgG2a and mouse IgG1 respectively (Southern Biotechnology Associates, Birmingham, AL, U S A.), in combination with TP3

Approximately 1-2 × 10^5 cells of the cell suspensions were washed in PBS, filtered to remove clumps and horny material, and resuspended in 500 µl of a solution with Vim3B4 diluted 1 50 and RKSE60 diluted 1 15 in PBS After incubation for 30 min at room temperature in the dark, the cells were washed in PBS containing 1% HINCS, resuspended and incubated for 15 min at 5°C in a solution of 500µl PBS, containing 2µl goat-anti-mouse-PE, 10µl goat-anti-mouse-FITC, 10µl normal goat serum and 5 µl HINCS After a third washing step DNA staining was performed by addition of 300µl TP3 (1µmol/l in PBS) and 50 µl RNase (1 mg/ml in PBS) (Sigma, St Louis, MO, U S A)

Flow cytometric analysis

The flow cytometric measurements and analysis were performed before the treatment codes were revealed From each sample 5,000-10,000 gated cells were measured and analysed using an EPICS® Elite flow cytometer (Coulter, Luton, U K) equipped with a dual laser system PE and FITC were excited with an air-cooled argon ion laser (15 mW, 488 nm) TP3 was excited with a HeNe laser (10 mW, 633 nm) Fluorescence was measured using bandpass filters of 520-530 nm (green, FITC), 555-595 nm (orange, PE), and 670-680 nm (red, TP3) The area/peak ratio of the red signal (DNA) was used to discriminate between doublets of diploid cells (clumps) and real single tetraploid cells [25] After setting appropriate gates with the EPICS® Elite software percentages of vimentin- and keratin 10 positive cells were calculated Using Multicycle™ software (Phoenix Flow Systems, San Diego, CA, U S A) the percentages of basal keratinocytes in S- and G_2M-phase of the cell cycle (proliferation) were calculated from DNA histograms

Statistical analysis

Changes in the relative numbers of vimentin-positive cells, keratin 10 positive keratinocytes and basal cells in S- and G_2M-phase before and after treatment were analysed using the paired *t*-test for means (two-tail) Differences between therapies were assessed using the two-sample *t*-test assuming equal variances (two-tail) Analysis of the correlation between clinical and flow cytometric scores was performed by calculation of the Pearson correlation coefficient

RESULTS

Clinical response

Of 40 included patients 39 completed the present study. One subject was withdrawn at week 4 because of severe generalized itching and worsening of psoriasis. This patient had used calcipotriol cream twice daily for 3 weeks. All four therapy regimens resulted in a statistically significant reduction of PASI scores. Percentages were 50% (calcipotriol once daily, D), 64% (calcipotriol twice daily, DD), 58% (calcipotriol in combination with clobetasone, DC) and 55% (calcipotriol in combination with betamethasone, DB). Between the four therapies no statistically significant difference in the decrease of the PASI score could be demonstrated. Clearance of the test lesions was reached in 10 subjects: values for each therapy group were 2 (D), 1 (DD), 3 (DC) and 4 (DB). In 5 subjects even clearance of all lesions was reached. These patients all had used calcipotriol in combination with a corticosteroid (DC: 3, DB: 2).

Flow cytometric analysis

From one patient the initial keratotome biopsy proved to contain exclusively the most superficial layers of the epidermis. Therefore, flow cytometric assessment was performed on 76 epidermal cell suspensions obtained from test lesions of 38 patients.

The percentage of intact cells per sample (corrected for debris and clumps) (mean ± standard error of the mean) was 78·1 ± 1·2 (range 48·7 - 93·5). The clinical scores and the flow cytometric analyses of all biopsies before and after treatment are shown in Figure 1. It can be seen that the percentage of vimentin-positive cells correlates with the clinical expression of inflammation, i.e. erythema (r = 0·26, P < 0·05), that the relative number of keratin 10 positive keratinocytes (differentiation) correlates inversely with the score for desquamation (r = −0·40, P < 0·01), and that the percentage of basal keratinocytes in S- and G_2M-phase (proliferation) correlates with the induration score of the psoriatic test lesions (r = 0·53, P < 0·0001).

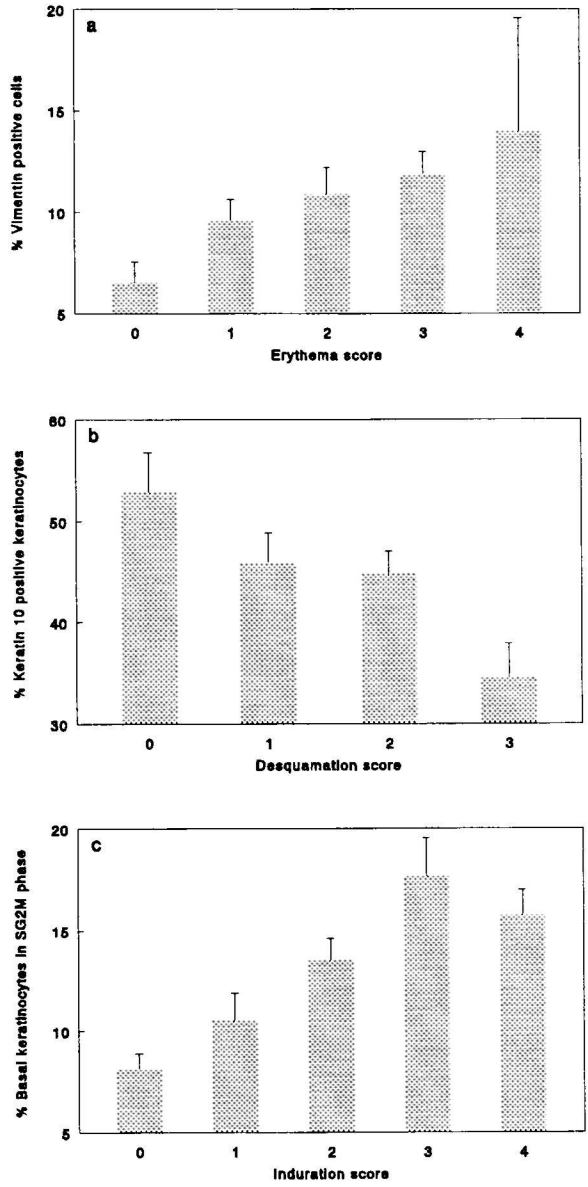


Figure 1. Correlation of clinical scores and flow cytometric analyses (means ± SEM) of all test lesions (n = 76). (a) Percentages of vimentin-positive cells vs. erythema score. (b) Percentages of keratin 10 positive keratinocytes vs. desquamation score. (c) Percentages of basal keratinocytes in S- and G_2M phase vs. induration score.

Table 1. Percentages of vimentin positive cells, keratin 10 positive keratinocytes and basal keratinocytes in S- and G_2M phase in epidermal cell suspensions prepared from keratotome biopsies taken from test lesions of psoriasis patients during treatment (means ± SEM)

		Vimentin		Keratin 10		S and G_2M	
		Baseline	After treatment	Baseline	After treatment	Baseline	After treatment
Calcipotriol once daily	n = 9	10 8 ± 2 0	8 5 ± 1 0	41 0 ± 5 3	50 2 ± 3 9	14 1 ± 2 3	10 8 ± 2 2
Calcipotriol twice daily	n = 9	10 9 ± 2 2	8 4 ± 1 1	44 3 ± 4 4	54 3 ± 4 1	15 2 ± 1 2	10 1 ± 1 9*
Calcipotriol / clobetasone 17-butyrate	n = 9	14 4 ± 1 8	7 8 ± 0 8*	35 7 ± 3 3	48 2 ± 5 3*	16 1 ± 2 5	8 9 ± 1 0**
Calcipotriol / betamethasone 17-valerate	n = 11	15 5 ± 2 8	10·7 ± 1 5	37 9 ± 4 2	40 5 ± 3 1	16 5 ± 2 3	8 7 ± 1 3**

*/** Significant differences (compared to baseline) at the P < 0 05 and P < 0 01 level, respectively

Table 1 summarizes the expression of the markers for proliferation, differentiation and inflammation before and after treatment. All therapy regimens except the once daily application of calcipotriol resulted in a statistically significant decrease of the percentage of basal keratinocytes in S- and G_2M-phase. Following calcipotriol once daily and twice daily the reductions of this parameter were 24% ($P = 0\ 24$) and 34% ($P < 0{\cdot}05$), respectively, whereas the reductions following treatment with calcipotriol/clobetasone 17-butyrate and calcipotriol/betamethasone 17-valerate were 44% ($P < 0{\cdot}01$) and 47% ($P < 0{\cdot}01$), respectively With respect to the number of vimentin-positive cells a statistically significant decrease was reached in the calcipotriol/clobetasone butyrate-treated group (47%, $P < 0{\cdot}05$). Values for the other therapy groups were 29% ($P = 0{\cdot}19$) for calcipotriol/betamethasone, 21% ($P = 0{\cdot}34$) for calcipotriol once daily and 23% ($P = 0{\cdot}42$) for calcipotriol twice daily The percentage of differentiated keratinocytes (keratin 10 positive) increased in all therapy groups. Statistical significance was only reached with calcipotriol/clobetasone butyrate (35%, $P < 0{\cdot}05$).

DISCUSSION

From the present flow cytometric evaluation the following conclusions were drawn: (i) twice daily treatment with calcipotriol cream results in a substantial decrease of the percentage of cells in S- and G_2M-phase in the basal cell layer, without a significant effect on the percentage of vimentin-positive cells; (ii) once daily treatment with calcipotriol cream does not result in a significant reduction of the percentage of cells in S- and G_2M-phase in the basal cell layer; (iii) combination of calcipotriol and topical steroids results in a more marked decrease of the epidermal proliferative activity compared to calcipotriol treatment; (iv) a marked reduction of the percentage of vimentin-positive cells is observed following the concurrent use of calcipotriol and topical steroids whereas calcipotriol monotherapy has virtually no effect on this marker, and (v) an increase of the relative number of differentiated keratinocytes is only reached after concurrent use of calcipotriol and corticosteroids.

In the present study the effect of the different treatment schedules was assessed using flow cytometric quantification of epidermal growth, and of markers for differentiation and inflammation. These markers were chosen in analogy to the clinical features of the psoriatic lesion, i.e. erythema, induration and desquamation that are assessed in the PASI score. In previous studies the methodology was evaluated in epidermal hyperproliferation induced by sellotape stripping,[26] after application of leukotriene B_4 to normal skin[27] and in psoriatic patients before and following treatment.[21] In Figure 1 the correlation between the percentage of basal cells in S- and G_2M-phase and induration, between the percentage of vimentin-positive cells and erythema, and between the percentage of keratin 10 positive keratinocytes and scaling further substantiates the relationship between clinical features of the psoriatic lesion and the cell biological equivalent.

In a previous immunohistochemic study it was shown that calcipotriol ointment twice daily had a minor effect on cutaneous inflammation.[9] In a recent study using the same flow cytometric analytical method it was shown that Tacalcitol® (1α,24 dihydroxyvitamin D_3, 4 μg/g) ointment, applied once daily, had a substantial effect on epidermal proliferation (34% reduction of the percentage basal keratinocytes in S- and G_2M-phase) without a significant effect on the percentage of vimentin-positive cells.[21] The present study on calcipotriol demonstrates a similar preponderance of the antiproliferative effect. As once daily treatment with calcipotriol cream had a minor effect on epidermal growth of the psoriatic lesion, twice daily application seems to be a more optimal approach.

Insight in the *in vivo* action of antipsoriatic treatments helps to define promising combination schedules. The present study indicates that concurrent use of calcipotriol and a topical corticosteroid has a more pronounced effect on epidermal hyperproliferation and inflammation, compared to monotherapy with calcipotriol. In this respect it is of interest that after clobetasol 17-propionate monotherapy, the reduction of the percentage of basal cells in S-and G_2M-phase was 72% and the reduction of the percentage of vimentin-positive was 62% (unpublished data). Surprisingly, a marked difference between both combination therapies was observed with respect to the expression of the differentiation-related keratin 10· the increase after treatment with calcipotriol and clobetasone 17-butyrate was 35% and following treatment with the combination of calcipotriol and betamethasone 17-valerate 7%.
In conclusion, the present flow cytometric study lends support for the hypothesis that the effect of calcipotriol once daily is inferior to calcipotriol twice daily and that the combination of calcipotriol and a corticosteroid has a better antipsoriatic efficacy compared to calcipotriol monotherapy.

ACKNOWLEDGEMENTS

We thank Leo Pharmaceutical Products (Denmark) for kindly supporting the present study.

REFERENCES

1 van de Kerkhof PCM Biological activity of vitamin D analogues in the skin, with special reference to antipsoriatic mechanisms Br J Dermatol 1995, 132 675-682

2 Binderup L, Bramm E Effects of a novel vitamin D_3 analogue MC 903 on cell proliferation and differentiation in vitro and calcium metabolism in vivo Biochem Pharmacol 1988, 37 889-895

3 Kragballe K, Wildfang IL Calcipotriol MC903, a novel vitamin D_3 analogue, stimulates terminal differentiation and inhibits proliferation of cultured human keratinocytes Arch Dermatol Res 1990, 282 164-167

4 Bagot M, Charue D, Lescs M-C, Ramphile R, et al Immunosuppressive effects of 1,25-dihydroxyvitamin D_3 and its analogue calcipotriol on epidermal cells Br J Dermatol 1993, 130 424-431

5 Muller K, Svenson M, Bendtzen K 1,25-dihydroxyvitamin D_3 and a novel vitamin D analogue MC903 are potent inhibitors of human interleukin 1 *in vitro* Immunol Letters 1988, 17 361-366

6 de Mare S, de Jong EMGJ, van de Kerkhof PCM DNA content and K_s8 12 binding of the psoriatic plaque lesion during treatment with the vitamin D_3 analogue MC903 and betamethasone Br J Dermatol 1990, 123 291-295

7 Holland DB, Roberts SG, Russell A, Wood EJ, et al Changes in epidermal keratin levels during treatment of psoriasis with the topical vitamin D_3 analogue MC903 Br J Dermatol 1990, 122 284 (Abstract)

8 Berth-Jones J, Fletcher A, Hutchinson PE Epidermal cytokeratin and immunocyte responses during treatment of psoriasis with calcipotriol and betamethasone valerate Br J Dermatol 1992, 126 356-361

9 de Jong EMGJ, van de Kerkhof PCM Simultaneous assessment of inflammation and epidermal proliferation in psoriatic plaques during long-term treatment with the vitamin D_3 analogue MC903 modulations and interrelations Br J Dermatol 1991, 124 221-229

10 Oxholm A, Oxholm P, Staberg B, Bendtzen K Expression of interleukin-6-like molecules and tumour necrosis factor after topical treatment of psoriasis with a new vitamin D analogue (MC903) Acta Derm Venereol (Stockh) 1989, 69 385-390

11 Verburgh CA, Nieboer C Local application of vitamin D_3 derivative MC903 in psoriasis influence on cellular infiltrate, Langerhans cells and keratinocyte (KC) markers J Invest Dermatol 1989, 93 310 (Abstract)

12 Mallett RB, Coulson IH, Purkis PE, Leigh IM, et al An immunohistochemical analysis of the changes in the immune infiltrate and keratin expression in psoriasis treated with calcipotriol compared with betamethasone ointment Br J Dermatol 1990, 123 837 (Abstract)

13 Grossman RM, Thivolet J, Claudy A, Souteyrand P, et al A novel therapeutic approach to psoriasis with combination calcipotriol ointment and very low-dose cyclosporine Results of a multicenter placebo-controlled study J Am Acad Dermatol 1994, 31 68-74

14 Kragballe K Treatment of psoriasis by the topical application of the novel cholecalciferol analogue calcipotriol (MC 903) Arch Dermatol 1989, 125 1647-1652

15 Dubertret L, Wallach D, Souteyrand P, Perussel M, et al Efficacy and safety of calcipotriol (MC903) ointment in psoriasis vulgaris J Am Acad Dermatol 1995, 27 983-988

16 Kragballe K, Gjertsen BT, de Hoop D, Karlsmark T, et al Double-blind, right/left comparison of calcipotriol and betamethasone valerate in treatment of psoriasis vulgaris Lancet 1991, 337 193-196

17 Cunliffe WJ, Berth-Jones J, Claudy A, Fairiss G, et al Comparative study of calcipotriol (MC 903) ointment and betamethasone 17-valerate ointment in patients with psoriasis vulgaris J Am Acad Dermatol 1992, 26 736-743

18 Berth-Jones J, Chu AC, Dodd WAH, Ganpulle M, et al A multicentre, parallel-group comparison of calcipotriol ointment and short-contact dithranol therapy in chronic plaque psoriasis Br J Dermatol 1992, 127 266-271

19 van de Kerkhof PCM Vitamin D3 und seine Analoge in der Dermatologie Z Hautkr 1994, 69 219-226

20 van Hooijdonk CAEM, Glade CP, van Erp PEJ TO-PRO-3 iodide, a novel HeNe laser-excitable DNA stain as an alternative for propidium iodide in multiparameter flow cytometry Cytometry 1994, 17 185-189

21 Glade CP, van Erp PEJ, van Hooijdonk CAEM, Elbers ME, et al Topical treatment of psoriatic plaques with 1a,24 dihydroxyvitamin D_3 a multiparameter flow cytometrical analysis of

epidermal growth, differentiation and inflammation Acta Derm Venereol (Stockh) 1995, 75 381-385

22 Bauer FW, Boezeman JBM, van Engelen L, de Grood RM, et al Monoclonal antibodies for epidermal population analysis J Invest Dermatol 1986, 87 72-75

23 Ramaekers FCS, Puts JJ, Moesker O, Kant A, et al Antibodies to intermediate filament proteins in the immunohistochemical identification of human tumours an overview Histochem J 1983, 15 691-713

24 Leigh IM, Purkis PE, Whitehead P, Lane EB Monospecific monoclonal antibodies to keratin 1 carboxy terminal (synthetic peptide) and to keratin 10 as markers of epidermal differentiation Br J Dermatol 1993, 129 110-119

25 Bauer FW, Boezeman JBM Flow cytometric methods in human skin with respect to cell cycle kinetics In Psoriasis Cell Proliferation (Wright NB, Camplejohn RS, eds), Edinburgh Churchill Livingstone, 1983, 104-116

26 Glade CP, Seegers BAMPA, Meulen EFJ, van Hooijdonk CAEM, et al Multiparameter flow cytometric characterization of epidermal cell suspensions prepared from normal and hyperproliferative skin using an optimized thermolysin-trypsin protocol Arch Dermatol Res 1996, 288 203-210

27 Glade CP, Botermans RJG, van Erp PEJ, van de Kerkhof PCM The dynamics of the response of normal skin to single and multiple epicutaneous leukotriene B_4 applications analysed by three-colour flow cytometry Acta Derm Venereol (Stockh) 1995, 75 437-441

4.3 Flow cytometric assessment of clearance and relapse characteristics in psoriasis vulgaris after treatment with weekly clobetasol lotion under occlusion versus twice daily clobetasol ointment

Conrad P. Glade, Carine J.M. van der Vleuten, Piet E.J. van Erp,
Peter C.M. van de Kerkhof, Department of Dermatology, University Hospital Nijmegen, The Netherlands
to be submitted

SUMMARY

Clearance and relapse characteristics of clobetasol lotion under hydrocolloid occlusion once weekly versus clobetasol ointment twice daily were assessed in a comparative flow cytometric study. Quantitative analysis of markers for epidermal proliferation, differentiation and inflammation was performed on epidermal single cell suspensions prepared from 3 mm punch biopsies taken from 15 patients with psoriasis vulgaris before therapy, at clearance and 6 weeks after clearance. After treatment both therapy regimens resulted in substantial changes of all flow cytometric parameters, but clearance was induced earlier in the corticosteroid under hydrocolloid occlusion-treated group. With respect to the relapse phase no difference was observed between both treatments.

INTRODUCTION

Various studies showed the beneficial effect of hydrocolloid occlusive dressings (HCD) in the treatment of psoriasis vulgaris[1-3]. This mode of occlusion proved to interfere directly with epidermal growth and differentiation[1]. The combination of HCD with topical corticosteroids has a high therapeutic potential, up to normalization of the psoriatic skin[4]: Van de Kerkhof et al. showed that weekly treatment of psoriasis with triamcinolone lotion and HCD for 3 weeks resulted in total clearing in 20% of patients[5]. Juhlin et al. found clearance in all 15 patients treated with betamethasone lotion under HCD once weekly for 2 weeks. In the latter study the psoriatic lesions relapsed after 4-8 weeks[6]. Following treatment with clobetasol under HCD twice daily clinical healing was observed after 6-10 days and relapse occurred after 12 days[7].

Volden et al. demonstrated complete remission after 12 days of combined therapy with clobetasol lotion and HCD applied once weekly. The time until relapse was 1 to 6 months in 4 of 21 patients[8]. From these studies it can be concluded that a strong clinical response can be obtained with topical steroids in combination with HCD.
However, comparative studies with respect to clearance and relapse characteristics of topical corticosteroids without HCD versus the combination of topical corticosteroids with HCD have not been carried out so far. With respect to the duration of remission after treatment with topical corticosteroids under HCD, variations between 6-10 days and 6 months and even longer have been reported. In order to circumvent the variability of these clinical studies a quantitative characterization of the major cell biological processes before, during and following treatment might provide important information for the understanding of the dynamics of antipsoriatic therapy. As multiparameter flow cytometry permits simultaneous quantification of different cell parameters[9,10], we performed a quantitative flow cytometric analysis of markers for epidermal proliferation, differentiation and inflammation in patients who were treated with clobetasol ointment twice daily or clobetasol lotion once weekly under HCD. These patients participated in a multicentre clinical study on efficacy and posttreatment remission of both treatments[11]. Biopsies for flow cytometric analysis were taken at the Nijmegen centre before treatment, at clearance and 6 weeks after clearance. We addressed the following questions:
1) What are the flow cytometric characteristics of psoriatic lesions before treatment, after treatment with clobetasol ointment only and clobetasol lotion under HCD and 6 weeks after treatment?
2) Is there any difference between both therapy regimens?

MATERIALS AND METHODS

Patients, treatment and biopsies

Nineteen patients with psoriasis vulgaris were included and at random divided in two treatment groups. They had received no topical treatment for at least 2 weeks or systemic treatment for at least 2 months. All patients gave written consent. In each patient one test lesion was selected and treated with clobetasol ointment twice daily (n=9) or clobetasol lotion under HCD (Duoderm Extra Thin®, ConvaTec) once weekly (n=10) until clearance was observed or alternatively for a maximal period of 6 weeks. The definition of clearance was: erythema score 0 or 1 (5-point scale), no induration

and no desquamation. Patients visited the out-patient department every second week until a relapse occurred, i e clinical scores for erythema, induration and desquamation higher than 1,0,0 respectively For flow cytometric analysis from each patient 3 mm punch biopsies were taken before therapy, at clearance and 6 weeks after clearance

Cell isolation procedure

The preparation of epidermal single cell suspensions has been described by us before[12] In brief, the biopsy was washed in phosphate buffered saline (PBS) After overnight (16h) incubation at 4°C of the skin specimen with thermolysin (Sigma P-1512, St Louis, USA, 0 5 mg/ml dissolved in PBS with Ca^{++} and Mg^{++}, Seromed, Berlin, Germany) dermis and epidermis were separated with a fine forceps Then the remaining epidermis was incubated in 2 ml PBS containing 0 25 mg/ml trypsin (Sigma T-8253) and 3 0 mg/ml DTE (Sigma, D-8255) for 30 min at 37°C After adding 200 µl heat-inactivated newborn calf serum (HINCS, Life Technologies Ltd , Paisley, UK) the epidermis was gently mixed on a vortex to separate the keratinocytes resulting in a single cell suspension Then the stratum corneum was discarded After centrifugation the solution was resuspended The cells were fixed in 2 ml ice cold ethanol and kept at -20°C until staining and flow cytometric analysis

Triple labelling and flow cytometric analysis

A triple labelling was performed, using a DNA fluorochrome combined with two antibodies against intermediate filaments[10] To assess hyperproliferation, the DNA fluorochrome TO-PRO-3 iodide (TP3, Molecular Probes, Eugene, USA) was used TP3 intercalates with double string DNA and permits measurement of the proliferative activity of cells by quantification of the percentage of cells in S- and G_2M phase As TP3 also binds to RNA to some extent, it was used in combination with RNase To study inflammation Vim3B4 (Novocastra Laboratories Ltd , Newcastle upon Tyne, UK) was used This IgG2a-type mouse monoclonal antibody stains vimentin, an intermediate filament-type protein which occurs in mesenchymal cells i e polymorphonuclear neutrophils, lymphocytes, monocytes, macrophages, melanocytes and Langerhans' cells To quantify the differentiation process, the IgG1-type mouse monoclonal antibody RKSE60 (Dept of Mol Biology, University of Maastricht, The Netherlands) was used RKSE60 is directed against keratin 10, an intermediate filament-type protein that is expressed in differentiating keratinocytes Three-colour fluorescence was obtained with the fluorochromes fluorescein-isothiocyanate (FITC)

and phycoerythrin (PE) which are conjugated to monoclonal goat antibodies against mouse IgG2a and mouse IgG1 respectively (Southern Biotechnology Associates, Birmingham, USA), in combination with TP3

Approximately 1-2 × 10^5 cells of the cell suspensions were washed in PBS, filtered to remove clumps and horny material, and resuspended in 500 µl of a solution with Vim 3B4 diluted 1 50 and RKSE60 diluted 1 7 in PBS After incubation for 30 min at room temperature in the dark, the cells were washed in PBS containing 1% HINCS, resuspended and incubated for 15 min at 5°C in a solution of 500 µl PBS, containing 2 µl goat-anti-mouse-PE, 10 µl goat-anti-mouse-FITC, 10 µl normal goat serum and 5 µl HINCS After a third washing step DNA staining was performed by addition of 300 µl TP3 (1µM in PBS) and 50 µl RNase (1mg/ml in PBS) (Sigma, St Louis, USA)

From each sample 5,000-10,000 gated cells were measured and analysed using an EPICS® Elite flow cytometer (Coulter, Luton, UK) equipped with a dual laser system PE and FITC were excited with an air-cooled argon ion laser (15 mW, 488 nm) TP3 was excited with a HeNe laser (10 mW, 633 nm) Fluorescence was measured using bandpass filters of 520-530 nm (green, FITC), 555-595 nm (orange, PE), and 670-680 nm (red, TP3) The area/peak ratio of the red signal (DNA) was used to discriminate between doublets of diploid cells (clumps) and real single tetraploid cells After setting appropriate gates with the EPICS® Elite software percentages of vimentin- and keratin 10-positive cells are calculated Using Multicycle™ software (Phoenix Flow Systems, San Diego, USA) the percentages of basal and suprabasal keratinocytes and of non-keratinocytes in S- and G_2M phase (proliferation) of the cell cycle were calculated from DNA histograms

Statistical analysis

For comparison of paired values a paired two-sample t-test for means was used Unpaired data were analysed with a two-sample t-test assuming equal variances

RESULTS

Patients and biopsies

Of 19 patients, 15 completed the study One patient did not attend any visits after clearance of the test lesion and three discontinued treatment Of the patients who discontinued treatment, one developed a bacterial infection of the test lesion requiring additional therapy and for two the antipsoriatic response was unsatisfactory All

remaining 15 patients showed clearance after 6 weeks or less and visited the out-patient department every second week at least until a relapse had occurred In total forty-five punch biopsies were obtained for flow cytometric analysis

Clearing phase

The clinical response for both therapy regimens is summarized in Fig 1a The sum score is defined as the total of the erythema-, induration- and desquamation score of each test lesion As well before as after treatment both therapy groups were not statistically different with respect to the sum score The mean time until clearance was 18 5 days in the clobetasol lotion-HCD treated group (n=9) and 28 days in the clobetasol ointment group (n=6)

The results of the flow cytometric analysis are shown in Fig 1b-d Both the lotion under HCD as the ointment regimen resulted in a significant decrease of proliferative activity of the basal compartment ($p<0.0001$ and $p<0.01$) The amount of vimentin-positive cells was substantially reduced ($p<0.005$ and $p<0.02$) With respect to epidermal differentiation the percentage of keratin 10-positive keratinocytes increased ($p<0.0003$ and $p<0.001$) No statistically significant difference was observed between the two therapies With one exception the flow cytometric values showed no significant difference between the two therapies Only before therapy the amount of vimentin-positive cells was higher in the lotion-HCD-treated group compared to the clobetasol ointment group ($p<0.05$)

No correlation could be demonstrated between flow cytometric parameters before therapy and the clearance time

Relapse phase

Thirteen out of 15 patients showed a clinical relapse after 6 weeks The mean time until relapse was 29 days after the discontinuation of the treatment with clobetasol lotion under HCD One patient in the latter group remained without relapse After treatment with clobetasol ointment the mean time until relapse was 37 5 days The mean clinical sum score 6 weeks after clearance was 4 1 in the lotion under HCD-treated group and 3 2 in the ointment-treated group (Fig 2a) Compared to the corresponding scores at clearance these changes were statistically significant ($p<0.002$ and $p<0.01$, respectively)

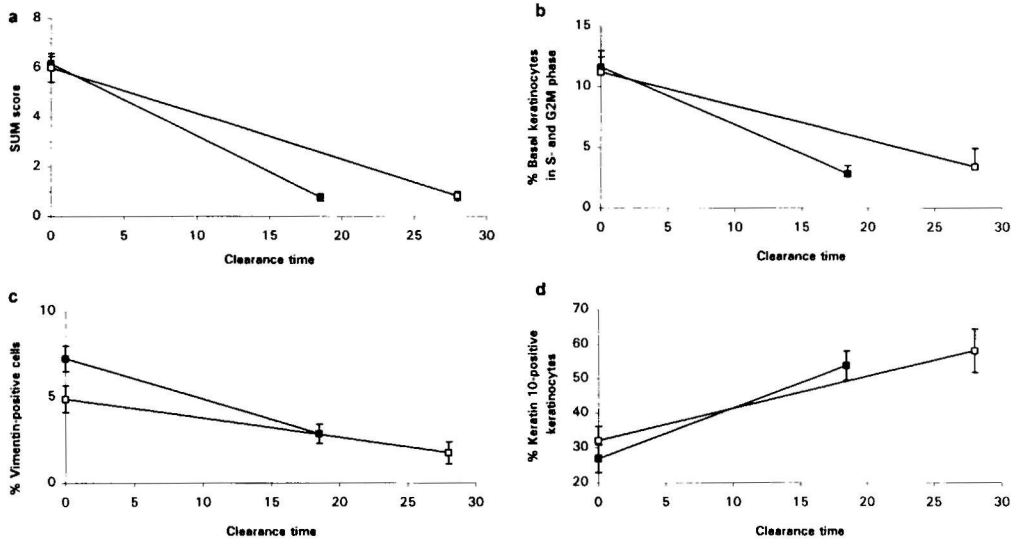


Figure 1a-d. Clinical sum scores (**a**) and flow cytometric scores (**b-d**) (mean ± SEM) of psoriatic lesions before treatment and at clearance. Values are given for weekly applications of clobetasol lotion under HCD (■) versus twice daily applications of clobetasol ointment (□).

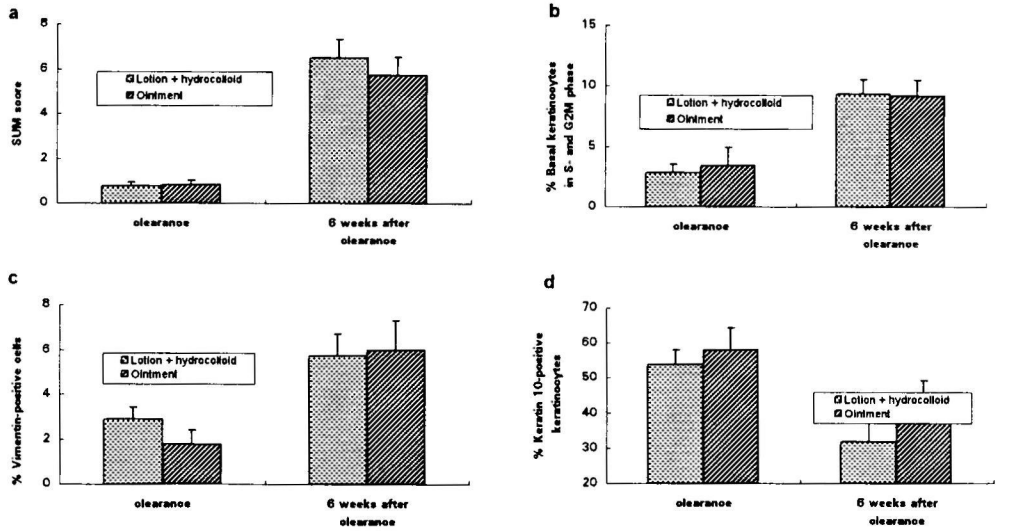


Figure 2a-d. Clinical sum scores (**a**) and flow cytometric scores (**b-d**) (mean ± SEM) of psoriatic lesions at clearance and 6 weeks after clearance. Values are given for weekly clobetasol lotion under HCD versus twice daily clobetasol ointment.

In Fig. 2b-d the results of the flow cytometric analysis are summarized. Compared to the situation at clearance the mean proliferative activity was substantially increased 6 weeks after clearance in both lotion-HCD and ointment-treated lesions ($p<0.002$ and $p<0.05$). The number of vimentin-positive cells present in the epidermal compartment increased in both therapy groups ($p<0.03$ and $p<0.05$). The percentage of keratin 10-positive keratinocytes decreased ($p<0.01$ and $p=0.17$). Statistical analysis of the flow cytometric values 6 weeks after clearance revealed no difference between both therapies.

DISCUSSION

In the present study a flow cytometric analysis of clearance and relapse characteristics of psoriatic lesions after treatment with clobetasol lotion under HCD once weekly versus clobetasol ointment twice daily is presented. In all patients both therapy regimens resulted in clearance of the test lesions. However, clinical clearance was reached earlier in lesions under occlusion. For both therapies the flow cytometric assessment revealed substantial changes in cell biological parameters during and after therapy. In this respect no difference was demonstrated between both therapies.

In Table 1 the mean flow cytometric values for all 15 lesions are compared. With respect to epidermal proliferation at clearance a strong reduction of the number of basal keratinocytes in S- and G_2M phase is demonstrated. The value of 3.1% is even lower than the value of 5.5% which represents the proliferative activity of normal skin[12]. In 4 out of 15 patients these percentages ranged from 0.1 to 0.5%. This means that after treatment DNA synthesis was almost totally suppressed in some patients. These data correspond with Goodwins[13], who investigated thymidine uptake of lesional and uninvolved psoriatic skin before and after treatment with topical corticosteroids under polyethene occlusion. Like in the present study, he described a totally suppressed uptake in some patients and a rebound in all patients after treatment had been stopped. In some of these patients DNA synthesis was also totally suppressed in the uninvolved skin. As Baxter et al.[14] and Fry et al.[15] both observed a decreased mitotic index in psoriatic lesions after treatment with occlusive dressings without adjunct treatment, the reduced proliferative activity is not exclusively a result from the application of corticosteroids. The present study, showing the relative fast clearance in the lesions treated with the combination of clobetasol lotion and HCD compared to the

lesions treated with clobetasol ointment monotherapy illustrates the additional value of HCD in treating psoriasis.

At clearance of the test lesions the amount of keratin 10-positive keratinocytes reached values present in normal skin [12]. Such suggests that after treatment a normalization of the psoriatic epidermis was reached with respect to differentiation. This effect is not only due to the topical corticosteroids. In an immunohistochemical study Gerritsen et al.[1] demonstrated that filaggrin and involucrin expression (markers for late keratinization) increased substantially in psoriatic plaques after application of HCD for 3 weeks.

Table 1. Comparison of flow cytometric values (mean ± SEM, n=15) of epidermal single cell suspensions prepared from 3 mm punch biopsies taken from all test lesions before therapy, at clearance and 6 weeks after clearance.

	Before therapy	At clearance	6 weeks after clearance
% Basal keratinocytes in S- and G_2M phase	11 ± 0.9	3.1 ± 0.7	9.3 ± 0.9
% Keratin 10-positive keratinocytes	29 ± 2.9	56 ± 3.4	36 ± 5.0
% Vimentin-positive cells	6.3 ± 0.6	2.4 ± 0.4	5.8 ± 0.7

The intermediate filament protein vimentin is expressed in cells from mesenchymal origin. Therefore, in psoriasis vimentin occurs in the epidermal non-keratinocytes, i.e. lymphocytes, polymorphonuclear neutrophils, monocytes, macrophages, melanocytes and Langerhans' cells. After both therapy regimens the amount of epidermal non-keratinocytes decreased substantially, resulting in values even lower compared to normal skin[12]. As immunological parameters remained unaffected during HCD treatment of psoriatic plaques[1] the reduction of vimentin-positive cells in the psoriatic epidermis in the present study are totally due to the anti-inflammatory properties of the corticosteroids. A more moderate reduction of the amount of vimentin-positive cells in psoriatic epidermis is observed after treatment with several vitamin D_3 analogues. After 6 weeks treatment with 1α,24 dihydroxyvitamin D_3 ointment (4μg/g once daily) a reduction of 20% was obtained[10]. After treatment with 8 weeks calcipotriol (50 μg/g twice daily) this percentage was in the same range: 23 % (data on file).

In conclusion, flow cytometric analysis demonstrated a strong interference of two different antipsoriatic therapy regimens with epidermal proliferation, differentiation

and inflammation. The combination of clobetasol lotion once weekly with HCD shortened the period until clearance was reached compared to clobetasol ointment twice daily The remission time after clearance was not substantially different for both therapies. Also the assessment of flow cytometric parameters 6 weeks after clearance showed comparable patterns. This indicates that with respect to relapse characteristics the use of topical corticosteroids under HCD is comparable to the classical corticosteroid treatment.

ACKNOWLEDGEMENTS

The cell biological results described in this communication were derived from patients who also participated in a larger multicentre comparative study (ConvaTec, data on file). The authors wish to acknowledge Prof Dr. K. Kragballe, Prof. Dr. G. Volden, Dr. R White, K. Åberg and B. Willemsteijn for helpfull discussion. The authors wish to thank ConvaTec for financial support of this study.

REFERENCES

1 Gerritsen MJP, van Vlijmen-Willems IMJJ, Chang A, van de Kerkhof PCM The effect of a hydrocolloid occlusive dressing (DuoDERM E) on keratinization in psoriasis vulgaris Acta Derm Venereol (Stockh) 1994, 74 483-484

2 Friedman SJ Management of psoriasis vulgaris with a hydrocolloid occlusive dressing Arch Dermatol 1987, 123 1046-1052

3 Telfer NR, Ryan TJ, Blanc D, Merk H, et al Results of a multicentre trial of actiderm in the treatment of of plaque psoriasis In Beyond occlusion· dermatology proceedings (Ryan TJ, ed), London, New York Royal Society of Medicine Services Limited, 1988, 53-58

4 Broby-Johansen U, Karlsmark T, Petersen LJ, Serup J Ranking of the antipsoriatic effect of various topical corticosteroids applied under a hydrocolloid dressing - skin-thickness, blood-flow and colour measurements compared to clinical assessments Clin Exp Dermatol 1990, 15 343-348

5 van de Kerkhof PCM, Chang A, van der Walle HB, van Vlijmen-Willems IMJJ, et al Weekly treatment of psoriasis with a hydrocolloid dressing in combination with triamcinolone acetonide Acta Derm Venereol (Stockh) 1994, 74 143-146

6 Juhlin L Treatment of psoriasis and other dermatoses with a single application of a corticosteroid left under a hydrocolloid occlusive dressing for one week Acta Derm Venereol (Stockh) 1989, 69 355-357

7 Schubert C, Christophers E Mast cells and macrophages in early relapsing psoriasis Arch Dermatol Res 1985, 277 352-358

8 Volden G Successful treatment of chronic skin diseases with clobetasol propionate and a hydrocolloid occlusive dressing Acta Derm Venereol (Stockh) 1992, 72 69-71

9 van Erp PEJ, Rijzewijk JJ, Boezeman JBM, Leenders J, et al Flow cytometric analysis of epidermal subpopulations from normal and psoriatic skin using monoclonal antibodies against intermediate filaments Am J Pathol 1989, 135 865-870

10 Glade CP, van Erp PEJ, van Hooijdonk CAEM, Elbers ME, et al Topical treatment of psoriatic plaques with 1a,24 dihydroxyvitamin D_3 a multiparameter flow cytometrical analysis of epidermal growth, differentiation and inflammation Acta Derm Venereol (Stockh) 1995, 75 381-385

11 Volden G, Kragballe K, van de Kerkhof PCM, White R, et al Clearance and posttreatment remission of once weekly treatment with clobetasol-17-propionate lotion under actiderm and twice daily treatment with clobetasol-17-propionate ointment in patients with psoriasis vulgaris (Manuscript in preparation)

12 Glade CP, Seegers BAMPA, Meulen EFJ, van Hooijdonk CAEM, et al Multiparameter flow cytometric characterization of epidermal cell suspensions prepared from normal and hyperproliferative skin using an optimized thermolysin-trypsin protocol Arch Dermatol Res 1996, 288 203-210

13 Goodwin P The effect of corticosteroids on cell turnover in the psoriatic patient Br J Dermatol 1976, 94 95-100

14 Baxter DL, Stoughton RB Mitotic index of psoriatic lesions treated with anthralin, glucocorticosteroid and occlusion only J Invest Dermatol 1970, 54 410-412

15 Fry I, Almeyda J, McMinn RMH Effect of plastic occlusive dressings on psoriatic epidermis Br J Dermatol 1970, 82 458-462

Chapter 5

GENERAL DISCUSSION

5.1 Multiparameter flow cytometry as a tool to evaluate antipsoriatic therapy

Conrad P. Glade, Piet E.J. van Erp, Jan B.M. Boezeman, Peter C.M. van de Kerkhof, Department of Dermatology, University Hospital Nijmegen, The Netherlands.

in press (Br J Dermatol)

SUMMARY

Objective comparison of different antipsoriatic therapies requires quantitative assessment of disease severity. However, clinical assessment with the widely used Psoriasis Area and Severity Index (PASI) introduces inaccuracy. An alternative is the quantitative analysis of different epidermal cell parameters using multiparameter flow cytometry. Our aim in the present study was to compare the clinical and flow cytometric approach to monitor disease activity and to evaluate antipsoriatic efficacy. Clinical scores for erythema, induration and scaling were assessed and biopsies for flow cytometric analysis were obtained from the psoriatic plaques of 89 patients before and after treatment with different therapeutic regimens consisting of vitamin D_3 analogues and corticosteroids. In total, 219 epidermal cell suspensions were analysed using triple-labelling, with the simultaneous staining of markers for epidermal proliferation (DNA dye TO-PRO-3), differentiation (antikeratin 10), and inflammation (antivimentin).

Correlation analysis was performed on 166 paired values obtained from 83 patients. A highly significant correlation was observed between erythema and the percentage of vimentin-positive cells, between scaling and the percentage of keratin 10-positive keratinocytes, and between induration and the number of basal keratinocytes in S- and G_2M phase, when all 166 biopsies were assessed. The latter correlation remained in the same range if the analysis was restricted to the 83 pretreatment biopsies. In contrast to the clinical scores, the flow cytometric analysis permitted a clear separation between the antiproliferative and anti-inflammatory or keratinization-enhancing effects of antipsoriatic treatment. The vitamin D_3 analogues proved to exert a mainly antiproliferative effect. The combination of calcipotriol and a topical corticosteroid improved all cell biological markers substantially, and clobetasol monotherapy had a powerful effect on these markers. In conclusion, multiparameter flow cytometry has

been shown to be a sensitive tool to evaluate the growth inhibiting, anti-inflammatory and keratinization-enhancing effects of antipsoriatic therapies.

INTRODUCTION

In the evaluation of antipsoriatic therapies, quantitative assessment of disease severity is essential. A popular and frequently-used method for clinical assessment is the Psoriasis Area and Severity Index (PASI). In this scoring system, described by Fredriksson and Pettersson[1], the percentage involvement and degree of erythema, induration and desquamation is estimated in four body areas. Using a formula, an ordinal value between 0 and 72 can be calculated. However, the authors stated that the PASI score should not to be regarded as an "exact" numerical value, since the severity rating is subjective. Marks *et al*[2] demonstrated a wide inter-observer error of even well-designed clinical assessment techniques, especially with respect to the calculation of the involved areas. Various groups have emphasized the potential for reaching inaccurate conclusions when clinical assessment alone is used[3,4]. Therefore, there is clearly a need for objective measurements of psoriasis severity.

Flow cytometry permits quantitative analysis of different cell parameters and can be applied to epidermal single-cell suspensions prepared from normal and diseased skin[5]. In analogy to the clinical signs which reflect the pathological changes in psoriasis, we developed a triple-labelling procedure with simultaneous staining of markers for epidermal proliferation (DNA content), differentiation (anti-keratin 10), and inflammation (anti-vimentin)[6,7]. Recently, with the development of a dermo-epidermal separation method using thermolysin, it has become possible to perform this flow cytometric procedure on cell suspensions prepared from 3mm punch biopsies[8].

In the present study we have analysed 219 epidermal single cell suspensions prepared from keratotome and punch biopsies of psoriatic plaques from 89 patients before and after treatment with different therapy regimens consisting of vitamin D_3 analogues and topical corticosteroids. Our aim in the study was to compare the clinical and flow cytometric approaches in monitoring disease activity and to establish whether flow cytometry is a valuable tool to evaluate and compare antipsoriatic therapies. We addressed in particular: to what extent the clinical scores for erythema, induration and desquamation correlate with the percentage of vimentin-positive cells, the percentage of basal keratinocytes in S- and G_2M phase and the percentage of keratin 10-positive keratinocytes, to what extent the improvement of clinical parameters reflects a change

in the cell biological markers, whether the method of preparation of cell suspensions influences the association of clinical and flow cytometric scores, and whether clinical and flow cytometric scores vary with different antipsoriatic therapies.

MATERIALS AND METHODS

Patients, treatment regimens and biopsy procedures

Data from 89 patients in total with psoriatic plaques were analysed. Patients' details have been described in our flow cytometric studies on the treatment of psoriatic plaques with 1α,24 dihydroxyvitamin D_3 ointment (Tacalcitol®), 4μg/g applied once daily for 8 weeks or placebo,[7] calcipotriol, 50μg/g once daily, twice daily or in combination with the topical corticosteroids clobetasone 17-butyrate. 0.5mg/g, and betamethasone 17-valerate, 1mg/g, for 8 weeks[9], and after treatment with clobetasol 17-propionate (unpublished data). Before the wash-out period of 2 weeks, representative psoriatic lesions were selected. Clinical scores for erythema, induration and desquamation of these test lesions were assessed, on a 5-point scale, before and after treatment. Scoring was performed by one investigator only, to avoid the interobserver error described by Marks *et al*[2]. Skin samples of the selected test were obtained simultaneously with clinical scoring. In this way 219 single-cell suspensions, derived from 152 keratotome biopsies and 67 punch biopsies, were prepared for flow cytometric analysis.

Single-cell suspensions and staining

Epidermal cell suspensions from keratotome biopsies were prepared using a trypsin separation method. The cell isolation procedure on punch biopsies consisted of dermo-epidermal separation with thermolysin and subsequent incubation with trypsin. In flow cytometric studies of epidermis, the quality of cell suspensions is essential. As enzymatic and mechanic methods are used to separate the cells, membrane antigens are easily damaged. Therefore, we decided to use a multiparameter technique with simultaneous quantification of the DNA content and of the intermediate filaments. These filaments are resistant to breakdown and antibodies are well-studied. Several keratins are highly expressed in the epidermis. Keratin 10 is a well-known epidermal differentiation marker. Vimentin is expressed in all epidermal mesenchymal cells, i.e dendritic cells and (especially in psoriatic skin) inflammatory infiltrate cells. A detailed description of both cell isolation methods and the staining procedure for

multiparameter flow cytometric analysis have been published[8]. Cells after isolation were fixed in 70% w/v ethanol until required for staining.

Multiparameter flow cytometry by the simultaneous measurement of fluorescein-isothiocyanate (FITC), phycoerythrin (PE) and propidiumiodide (PI) is considered difficult because of the large spectral overlap of PE and PI. Hoechst 33342 and 7AAD have been proposed as alternatives to PI in multiparameter flow cytometry. However, in our hands, 7AAD gave unacceptably high coefficients of variation (CVs). Hoechst 33342 staining requires an expensive ultraviolet laser, which is usually not present in commercial flow cytometers. On the contrary, many research flow cytometers are equipped with a low-power HeNe laser (633 nm), which excites TO-PRO-3 iodide (TP3) (peak absorbance at 642 nm). The quality of staining with TP3 has been extensively studied by our group[6]. At a concentration of 1 μmol/l, typical cell cycle histograms of peripheral blood lymphocytes showed a CV of 3.6%. The determination of human and mouse cell lines S-phase fractions with TP3 and PI showed a good correlation. Combined with FITC and PE, TP3 was superior than PI as a DNA stain in multiparameter flow cytometry.

Spectral overlap of the fluorochromes used proved to be minimal. After a first incubation for 30 min with anti-vimentin (Vim3B4, mouse IgG2a, Novocastra Laboratories Ltd., Newcastle upon Tyne, U.K.) and anti-keratin 10 (RKSE60, mouse IgG1, Department of Molecular Biology, University of Maastricht, The Netherlands) a second incubation for 15 min with the fluorochromes FITC and PE conjugated to monoclonal goat antibodies against mouse IgG2a and mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL, U.S.A.), respectively, was performed. After a third washing step, DNA staining was performed by addition of 300μl TP3 (1μmol/l in phosphate buffered saline (PBS)) (Molecular Probes, Eugene, OR, U.S.A.) and 50μl RNAase (1mg/ml in PBS) (Sigma, St. Louis, MO, U.S.A.).

Flow cytometric analysis

The flow cytometric analysis of triple-stained cell suspensions has been described before[7,8]. From each sample, 5,000-10,000 gated cells were measured and analysed using an EPICS® Elite flow cytometer (Coulter, Luton, U.K.) equipped with a dual laser system. PE and FITC were excited with an air-cooled argon ion laser (15mW, 488nm). TP3 was excited with a HeNe laser (10mW, 633nm). Fluorescence was measured using bandpass filters of 520-530nm (green, FITC), 555-595nm (orange, PE), and 670-680nm (red, TP3). The area/peak ratio of the red signal (DNA) was used

to discriminate between doublets of diploid cells (clumps) and real single tetraploid cells[10]. After setting appropriate gates with the EPICS® Elite software, the percentages of vimentin-positive cells, differentiated keratinocytes (vimentin-negative and keratin 10 positive) and basal keratinocytes (vimentin-negative and keratin 10 negative) were calculated. Using Multicycle™ software (Phoenix Flow Systems, San Diego, CA, U.S.A.) the percentages of basal keratinocytes in the S- and G_2M phases of the cell cycle (proliferation) were calculated from DNA histograms.

Statistic analysis

Analysis of the correlation between clinical and flow cytometric scores was performed by calculation of the Pearson correlation coefficient. In the placebo-controlled 1α,24 dihydroxyvitamin D_3 study four biopsies were obtained per patient and the corticosteroidmonotherapy study three biopsies were obtained per patient. Therefore, to avoid within-person variability, correlation analysis was restricted to paired values, i.e. before and after treatment, with omission of the values of the placebo-treated lesions. This resulted in 166 paired values obtained from 83 patients. To compare the flow cytometric scores before treatment for both cell isolation procedures, the paired t-test for means (two-tail) was used.

RESULTS

In all the 219 investigated flow cytometric samples, the number of intact cells was at least 30% (without debris and clumps). Coefficients of variation of the G1-peaks were ≤ 10. In Figure 1 we show the results of the correlation-analysis of clinical and flow cytometric scores, which was restricted to 166 paired values of 83 patients. It can be seen that high clinical scores for erythema correlate with high percentages of vimentin-positive cells (Fig. 1a; correlation coefficient $r = 0.41$, $P < 0.0001$), that the desquamation score inversely correlates with the number of keratin 10-positive keratinocytes (Fig.1b; $r = -0.44$, $P < 0.0001$) and that the induration of a psoriatic plaque correlates with its proliferative activity (Fig.1c; $r = 0.66$, $P < 0.0001$). High correlation coefficients were shown between the clinical scores of the psoriatic plaques: 0.79 (erythema and desquamation), 0.84 (erythema and induration), and 0.87 (induration and desquamation) with $P < 0.0001$ (Table 1). However, the coefficients of the flow cytometric values were -0.38, $P < 0.0001$ (vimentin and keratin 10); 0.37, $P < 0.0001$ (vimentin and SG_2M) and -0.13, (not significant) (SG_2M and keratin 10).

To exclude a possible effect of the treatment regimen on the degree of correlation, analysis of coefficients was also performed on values from biopsies obtained before treatment (n = 83). Table 1 shows that the correlation coefficients of clinical scores of untreated lesions only were reduced to 0.23 ($P < 0.05$, erythema and desquamation), 0.54 ($P < 0.0001$, erythema and induration), and 0.52 ($P < 0.0001$, induration and desquamation), whereas the correlations between the flow cytometric values remained below the level of statistical significance or strongly changed. Furthermore, it can be seen from Table 1 that, compared to the assessment on all lesions, the correlation in untreated lesions between basal cells in S- or G_2M phase, and induration, remained in the same range ($r = 0.52$; $P < 0.0001$). The association between erythema/vimentin and desquamation/keratin 10 was reduced to the lower values of 0.22 ($P < 0.05$) and -0.19 (not significant), respectively. To find out whether a change in clinical scores was reflected in changes in the flow cytometric scores, correlation analysis was performed on the differences of these scores before and after treatment. Figure 2 clearly shows that some degree of correlation exists. Quantitative analysis revealed correlation coefficients of $r = 0.14$ (not significant, Fig. 2a), $r = -0.28$ ($P < 0.01$, Fig 2b) and $r = 0.19$ ($P = 0.08$, Fig 2c).

Table 1. Correlation coefficients of clinical and flow cytometric scores (relative percentage of cells) of all psoriatic lesions ($n = 166$) and psoriatic lesions before treatment ($n = 83$)

	% Vimentin -positive	*P*	*% Keratin 10-positive*	*P*	*% basal cells in S- or G_2M-phase*	*P*	*Erythema*	*P*	*Induration*	*P*
n=166										
% Vimentin	1									
% Keratin 10	-0 38	<0 0001	1							
% SG_2M-phase	0.37	<0 0001	-0.13	0.087	1					
Erythema	0.41	<0.0001	-0.32	<0 0001	0 62	<0 0001	1			
Induration	0.44	<0 0001	-0 40	<0 0001	0 66	<0.0001	0 84	<0.0001	1	
Desquamation	0.37	<0 0001	-0.44	<0 0001	0 61	<0.0001	0 79	<0.0001	0.87	<0.0001
n=83										
% Vimentin	1									
% Keratin 10	-0.19	0 086	1							
% SG_2M-phase	0.084	0 45	0 25	0.021	1					
Erythema	0 22	0.044	0.16	0.14	0 44	<0 0001	1			
Induration	0 35	0 0010	-0 012	0 91	0 52	<0 0001	0 54	<0 0001	1	
Desquamation	0.093	0 40	-0 19	0.080	0 32	<0 0036	0 23	0 036	0.52	<0 0001

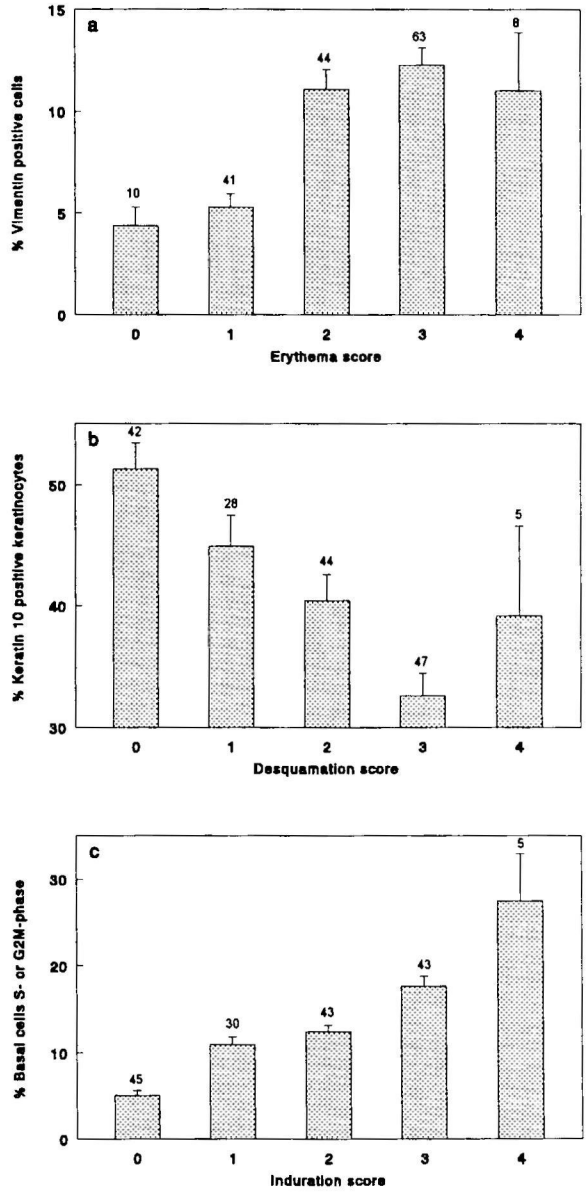


Figure 1a-c. Correlation of clinical scores and flow cytometric values (mean ± SEM) of 166 test lesions. The erythema score versus the percentage of vimentin-positive cells (**a**), the desquamation score versus the percentage of keratin 10-positive keratinocytes (**b**), and the induration score versus the percentage of basal keratinocytes in S- or G_2M-phase (**c**). The number of biopsies is indicated above the error bars.

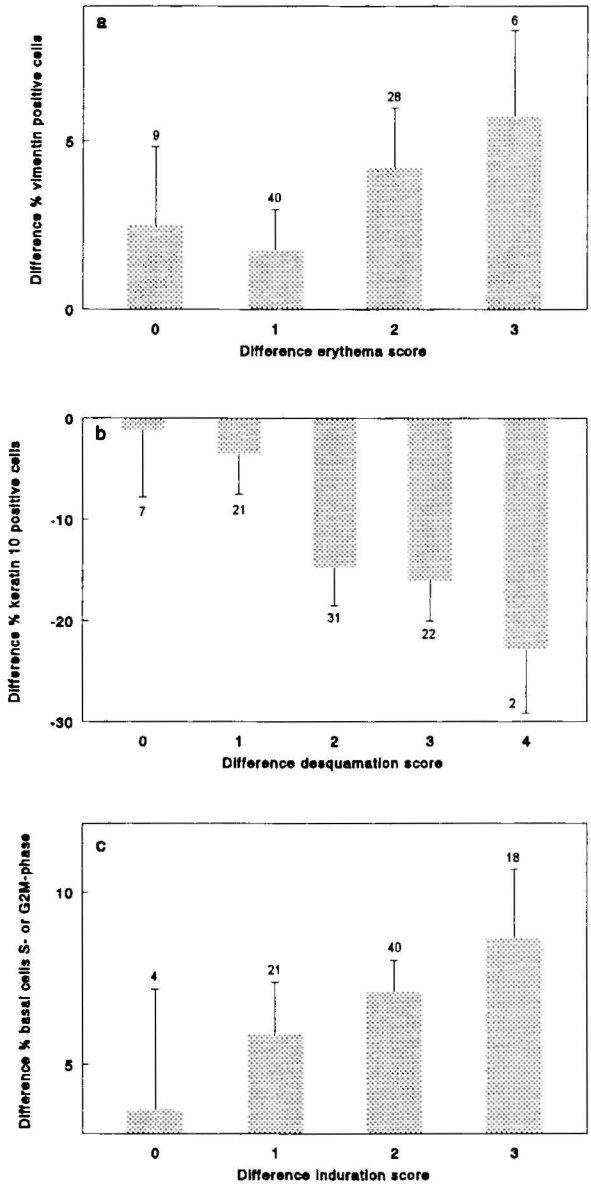


Figure 2a-c. Correlation of differences before and after treatment of clinical and flow cytometric parameters (mean ± SEM) of 83 test lesions. The erythema score versus the percentage of vimentin-positive cells (**a**), the desquamation score versus the percentage of keratin 10-positive keratinocytes (**b**), and the induration score versus the percentage of basal keratinocytes in S- or G_2M-phase (**c**). The number of biopsies is indicated above the error bars.

Table 2. Comparison of pretreatment flow cytometric values (relative percentage of cells) of punch and dermatotome biopsies prepared by different cell isolation procedures (n = 102) Δ implies the difference of values obtained by punch biopsies and dermatotome biopsies as a percentage of the value in dermatotome biopsies

$$(\Delta = \frac{[Dermatotome]-[Punch]}{[Dermatotome]} \times 100\%)$$

Clinical score		Flow cytometric score	Dermatotome	Punch	Δ (%)	P
Erythema	2	Vimentin (%)	17.4	6 4	63	< 0 001
	3		14 2	7 1	50	< 0 01
Induration	1	S- or G_2M-phase (%)	9 8	10 3	-5 1	N S
	2		14 2	9 3	35	< 0 01
	3		19 6	12 4	35	< 0 05
Desquamation	1	Keratin 10 (%)	50 3	29 8	41	< 0 05
	2		45 4	32 1	29	< 0 05
	3		35 2	26 6	24	< 0 05
	4		36 8	26 6	28	N S

In the present study, epidermal single-cell suspensions were prepared according to two different methods. Cell isolation of dermatotome biopsies (area 1 cm^2, thickness 0.4 mm) was obtained after a one-step trypsin incubation method. Three millimetre punch biopsies were processed using a two-step thermolysin-trypsin separation method. The question was addressed as to whether the cell isolation procedures influence the flow cytometric results. Therefore, in Table 2, the mean pretreatment flow cytometric values of each clinical score are given for both methods of cell isolation. It can be seen that all corresponding flow cytometric parameters (except for psoriatic plaques with an induration score of 1 and very scaly lesions were significantly lower, in epidermal cell suspensions prepared from punch biopsies, compared to dermatotome biopsies.

The results of the flow cytometric analysis, comparing both absolute and relative changes after different antipsoriatic therapy regimens and after placebo therapy, are given in Figure 3. With respect to epidermal proliferative activity (Fig. 3a, percentage basal keratinocytes in S and G_2M phase), 8 weeks of placebo treatment resulted in a reduction of 15% compared to the pretreatment situation. Both calcipotriol cream (50μg/g, applied twice daily) and 1α,24 dihydroxyvitamin D_3

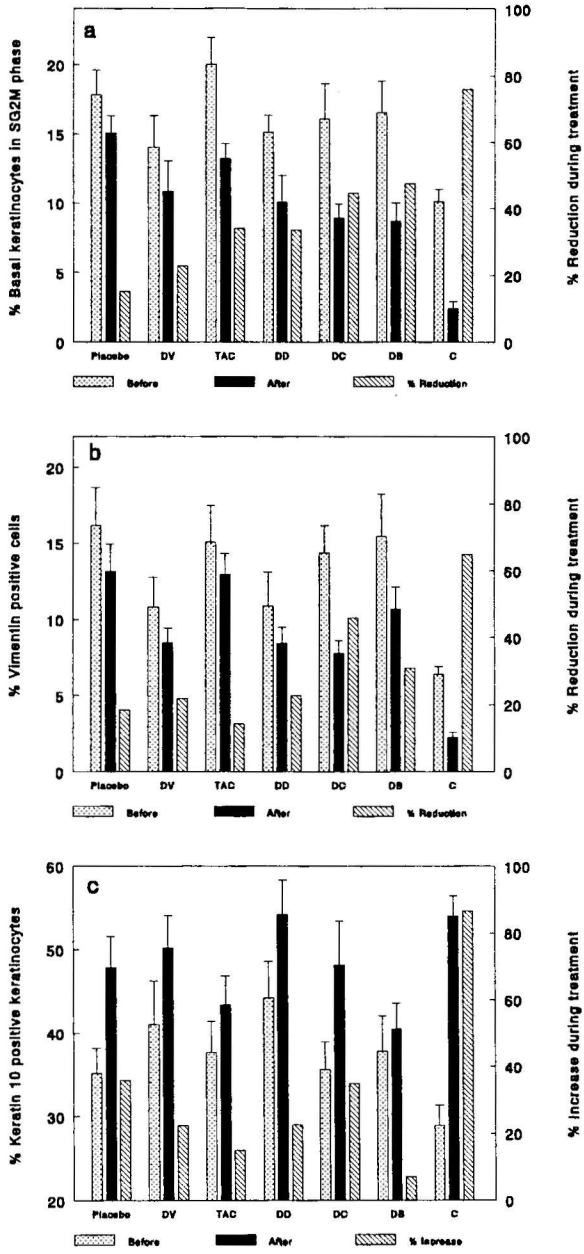


Figure 3a-c. Comparison of the absolute (mean ± SEM) and relative (%) changes of flow cytometric parameters after different antipsoriatic treatments with respect to the the percentage of basal keratinocytes in S- or G_2M-phase (**a**), the the percentage of vimentin-positive cells (**b**), and the percentage of keratin 10-positive keratinocytes (**c**). DV = calcipotriol once daily and vehicle once daily; TAC = Tacalcitol® (1α,24 dihydroxyvitamin D_3) ointment once daily; DD = calcipotriol twice daily; DC = calcipotriol once daily and clobetasone butyrate once daily; DB = calcipotriol once daily and betamethasone valerate once daily; C = clobetasol 17-propionate.

ointment (4μg/g, applied once daily) resulted in a reduction in proliferative activity of 34%. The application of calcipotriol cream 50μg/g once daily, however, resulted in a reduction of only 23%. The combination of topical corticosteroids and calcipotriol further increased the effect on epidermal proliferation. The reduction of proliferative activity in psoriatic plaques, treated for a maximum of 3 weeks with clobetasol propionate under hydrocolloid occlusion, proved to be 76%. With respect to the number of vimentin-positive cells (Fig. 3b) placebo treatment and monotherapy with the vitamin D_3 analogues resulted in a reduction of less than 23%. This is in contrast to treatment regimens comprising corticosteroids (combination or monotherapy), where a reduction of 31%-65% was observed. In Figure 3c, we indicate the effect of different antipsoriatic therapies on the number of keratin 10 positive keratinocytes (differentiation marker). Monotherapy with the vitamin D_3 analogues 1α,24 dihydroxyvitamin D_3 and calcipotriol (once daily or twice daily) resulted in an increase of 15%-23%. A combination of calcipotriol and topical steroids did not seem to increase this percentage substantially. In contrast, the monotherapy of psoriatic plaques with clobetasol propionate under occlusion increased the number of keratin 10 positive keratinocytes by 87%, compared to an increase of 36% for placebo.

In Figure 4 we summarize the effect of the different antipsoriatic therapies on the clinical scores of the test lesions. Therapy regimens using topical corticosteroids proved to have a more pronounced effect on erythema compared with monotherapy with vitamin D_3 analogues. The effect of placebo on the reduction of the erythema score was only 15% (Fig. 4a). All therapies showed a substantial reduction (50% or more) with respect to the induration score, in contrast to placebo (28%, Fig. 4b). The mean desquamation score was reduced by 42% after treatment with placebo, which implies that this parameter is placebo-sensitive. Mono or combination therapy using vitamin D_3 analogues showed reductions in desquamation values of 58%-81% (Fig. 4c).

DISCUSSION

A highly significant correlation was observed between erythema and the percentage of vimentin-positive cells, between scaling and the percentage of keratin 10-positive cells, and between induration and the percentage of basal cells in S- and G_2M phase, if all 166 biopsies are considered. The latter correlation remained equally pronounced,

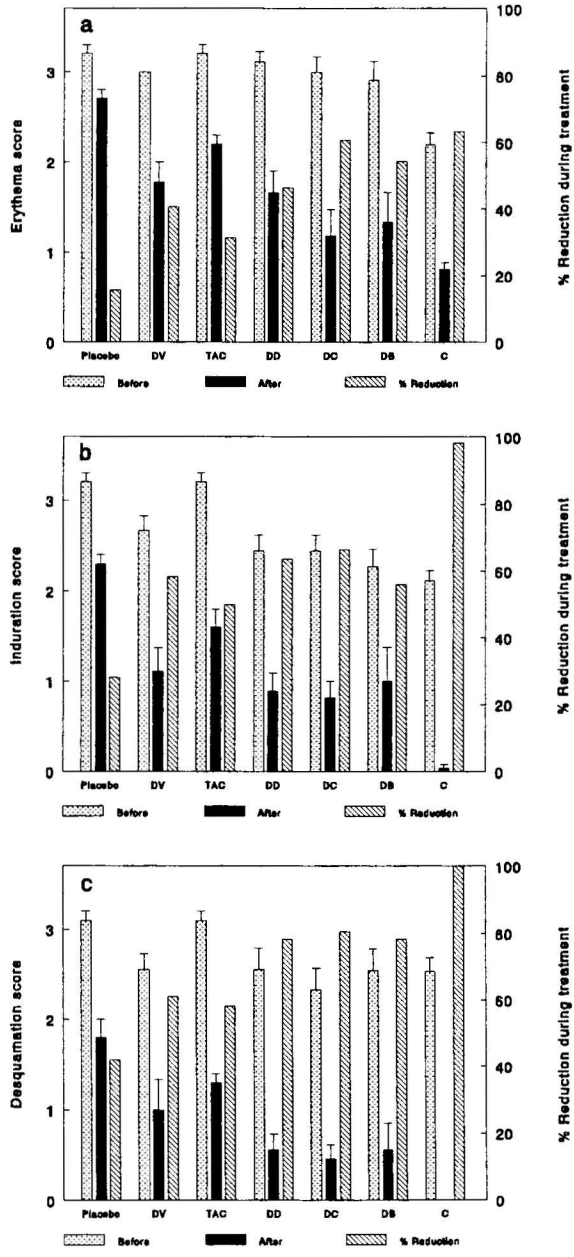


Figure 4a-c. Comparison of the absolute (mean ± SEM) and relative (%) changes of clinical scores after different antipsoriatic treatments with respect to erythema (**a**), desquamation (**b**), and induration (**c**).

whereas the other two associations were less prominent, if the analysis was restricted to 83 biopsies taken from untreated skin.

The percentage of vimentin-positive cells consists, to a large extent, of inflammatory cells. Although erythema, i.e. vasodilatation, is part of cutaneous inflammation, the appearance of inflammatory cells in the epidermis might have a different dynamic. In the marginal zone of spreading psoriatic plaques, significant changes in the microvasculature are visible well before the appearance of an inflammatory infiltrate[11,12]. Therefore, the incomplete correlation between the percentage of vimentin-positive cells in the epidermis and erythema can be explained by the partly independent dynamic of the microvasculature and the appearance of an infiltrate in the epidermis. Induration of the psoriatic plaque is supposed to be related to epidermal proliferation. Indeed, the high correlation between the percentage basal cells in S- and G_2M phase, and the induration score, is in line with this commonly held belief. As the formation of scale in the psoriatic plaque represents impaired differentiation, the distribution between the number of basal (keratin 10-negative) and suprabasal (keratin 10-positive) keratinocytes was presumed to be indicative of the desquamation score. However, due to the reduction of keratin 10 at some sites in the psoriatic lesion, this differentiation marker does not seem to visualize all suprabasal cells[13]. We also should reconcile the observation that keratin 10 is not a marker which includes all aspects of differentiation. Therefore, the correlation between scaling and keratin 10 expression has to be an incomplete one.

A description of the improvement in psoriatic plaques can be made clinically. The PASI score has been popular and practical, and adequate for comparative analysis. However, the interobserver variability is large[2]. It has been suggested by van de Kerkhof[4] that the scores of erythema, induration and scaling should be considered separately, as these markers provide information on the anti-inflammatory versus the antiproliferative capacity of treatment. The present study, however, suggests that the three scores are, to a large extent, correlated, which implies that the sum of these scores might be a more appropriate reflection of the psoriatic process. Such a sum might be the combined result of the cell biological denominator of the three processes. But we have to consider the possibility that this correlation, at least to some extent, is introduced by the unblinded investigator who, biased by a severe erythema, scaling or induration, might overinterpret the remaining signs. An important aspect of flow cytometric assessment during experimental treatment of psoriasis is the discrimination between antiproliferative, keratinization-enhancing and anti-inflammatory effects *in*

vivo. As demonstrated by correlation studies, the clinical parameters do not adequately permit this separation.

Vimentin can be used as a marker for epidermal infiltrate, showing higher values in psoriatic compared to normal skin. In Table 2, we compared pretreatment flow cytometric values of both separation methods in psoriatic skin, with equal erythema scores. As the epidermal infiltrate is assumed to be equal, and interference by treatment is excluded, the difference in the numbers of vimentin-positive cells may be accounted for by dermal infiltration. The data in Table 2 show that the biopsy and preparation procedure has a clear impact on the flow cytometric values reached. The objective for developing the dermo-epidermal separation method, using thermolysin on punch biopsies, was to obtain high quality cell suspensions with low dermal contamination and a high yield of basal cells[8]. Indeed, for each erythema score, the number of vimentin-positive cells is significantly lower in cell suspensions prepared from punch biopsies compared to dermatotome biopsies, indicating low dermal contamination. The suprabasal compartment (keratin 10-positive) is significantly smaller in cell suspensions prepared from punch biopsies, indicating the presence of a larger quantity of basal cells. It seems likely that a 0.4 mm dermatotome biopsy does not always contain all epidermal cells. Furthermore, in contrast to thermolysin, enzymatic separation with trypsin sometimes cleaves above the dermo-epidermal junction, resulting in a loss of basal cells. A lower proliferative activity in the basal compartment was found in the cell suspensions prepared from punch biopsies. This might be the result of less clumping in the cell suspension, as clumps mimick cells with a tetraploid DNA content. Another explanation for the lower percentage of basal cells in S- and G_2M phase might be the yield of a larger quantity of non- or slowly-proliferating stem cells, which have been suggested to be located at the tips of the rete ridges[14,15].

Although a comparison of different studies is hazardous due to preselection bias and subjective factors in clinical scoring, we have summarized the flow cytometric and clinical assessments of the efficacy of several topical antipsoriatic treatments in Figures 3 and 4). As different preparation methods were used, it is important to consider relative improvement only and to refrain from a comparison of absolute values. The placebo (the application of a bland emollient) had a substantial effect on the percentage of keratin 10-positive keratinocytes (a 36% increase in average counts). This effect on cell biology is in line with the tendency of the placebo to improve desquamation (by 42%), whereas induration and erythema are improved by 28% and

16%, respectively. Previously, we have demonstrated a reduction in the suprabasal expression of involucrin and the restoration of filaggrin expression during treatment with a hydrocolloid[16]. In the present study, we again suggest that improvement in the suprabasal compartment results from the artificial restoration of the skin barrier by an ointment.

The flow cytometric quantification reveals a comparable effect of calcipotriol, once or twice daily, with respect to the percentage of keratin 10-positive keratinocytes and the percentage of vimentin-positive cells. However, the reduction of basal cells in S- and G_2M phase was comparable for 1α,24 dihydroxyvitamin D_3 ointment (Tacalcitol®) once daily and calcipotriol twice daily, whereas once daily calcipotriol had a smaller effect on this proliferation marker. A comparison of clinical markers suggests that calcipotriol once and twice daily, and 1α,24 dihydroxyvitamin D_3 ointment once daily, induce an improvement well above that seen with placebo, with 1α,24 dihydroxyvitamin D_3 ointment tending to be slightly less effective. The twice daily application of calcipotriol resulted in a greater improvement in scaling. Monotherapy with vitamin D_3 analogues had a small effect on the percentage of vimentin-positive cells and the percentage of keratin 10-positive keratinocytes, at a level comparable with placebo. Interestingly, the addition of a topical corticosteroid to calcipotriol substantially enhanced the reduction of the percentage of vimentin-positive cells and, to a lesser extent, compensated for the abnormally low number of keratin 10-positive keratinocytes. Therefore, flow cytometric analysis suggests a powerful antipsoriatic effect for combination treatment of calcipotriol and a topical corticosteroid. This is, to some extent, expressed at the clinical level by the erythema score (see Fig. 4a). Clinical practice, however, has shown that the combination of calcipotriol and a topical corticosteroid has a major effect on recalcitrant psoriatic plaques. Treatment with a potent corticosteroid, such as clobetasol 17-propionate, is the most powerful topical antipsoriatic treatment available. Its effect on the percentage of basal keratinocytes in S- and G_2M phase, the percentage of vimentin-positive cells and the percentage of keratin 10-positive keratinocytes, is enormous. This effect is clearly seen clinically.

In conclusion, the clinical severity scores of psoriatic plaques are correlated to such an extent that they do not permit a separation between the effects on hyperproliferation, inflammation, and differentiation by antipsoriatic treatment. Triple-labelling flow cytometry permits the demonstration of antipsoriatic effects beyond the clinical scores, as assessed by the investigator, and further permits differentiation between the anti-

inflammatory, growth inhibiting and keratinization-enhancing capacity of antipsoriatic treatments

REFERENCES

1 Fredriksson T, Pettersson U Severe psoriasis - oral therapy with a new retinoid Dermatologica 1978, 157 238-44

2 Marks R, Barton SP, Shuttleworth D, et al Assessment of disease progress in psoriasis Arch Dermatol 1988, 125 235-40

3 Ramsay B, Lawrence CM Measurement of involved surface area in patients with psoriasis Br J Dermatol 1991, 124 565-70

4 van de Kerkhof PCM On the limitations of the psoriasis area and severity index (PASI)(letter) Br J Dermatol 1992, 126 205

5 van Erp PEJ, Rijzewijk JJ, Boezeman JBM, et al Flow cytometric analysis of epidermal subpopulations from normal and psoriatic skin using monoclonal antibodies against intermediate filaments Am J Pathol 1989, 135 865-70

6 van Hooijdonk CAEM, Glade CP, van Erp PEJ TO-PRO-3 iodide, a novel HeNe laser-excitable DNA stain as an alternative for propidium iodide in multiparameter flow cytometry Cytometry 1994, 17 185-9

7 Glade CP, van Erp PEJ, van Hooijdonk CAEM, et al Topical treatment of psoriatic plaques with 1a,24 dihydroxyvitamin D_3 a multiparameter flow cytometrical analysis of epidermal growth, differentiation and inflammation Acta Derm Venereol (Stockh) 1995, 75 381-5

8 Glade CP, Seegers BAMPA, Meulen EFJ, et al Multiparameter flow cytometric characterization of epidermal cell suspensions prepared from normal and hyperproliferative skin using an optimized thermolysin-trypsin protocol Arch Dermatol Res 1996, 288 203-10

9 Glade CP, van Erp PEJ, van de Kerkhof PCM Epidermal cell DNA content and intermediate filaments keratin 10 and vimentin after treatment of psoriatic plaques with calcipotriol cream once daily, twice daily and in combination with clobetasone 17-butyrate cream or betamethasone 17-valerate cream a comparative flow cytometric study Br J Dermatol 1996, 135 379-84

10 Bauer FW, Boezeman JBM Flow cytometric methods in human skin with respect to cell cycle kinetics In Psoriasis Cell Proliferation, Wright NB, Camplejohn RS, eds, Edinburgh Churchill Livingstone, 1983 104-16

11 MacDonald-Hull S, Goodfield M, Wood EJ, et al Active and inactive edges of psoriatic plaques identification by tracing and investigation by laser-Doppler flowmetry and immunocytochemical techniques J Invest Dermatol 1989, 92 782-5

12 de Jong EMGJ, Schalkwijk J, van de Kerkhof PCM Epidermal proliferation and differentiation, composition of the inflammatory infiltrate and the extracellular matrix in the margin of the spreading psoriatic lesion Eur J Dermatol 1991, 1 221-7

13 de Mare S, de Jong EMGJ, van Erp PEJ, et al Markers for proliferation and keratinization in the margin of the active psoriatic lesion Br J Dermatol 1990, 122 469-75

14 Lavker RM, Sun TT Epidermal stem cells J Invest Dermatol 1983 ,81 121s-7s

15. Potten CS, Morris RJ. Epithelial stem cells in vivo. J Cell Sci 1988; 10: 45-62.
16. Gerritsen MJP, van Vlijmen-Willems IMJJ, Chang A, et al. The effect of a hydrocolloid occlusive dressing (DuoDERM E) on keratinization in psoriasis vulgaris. Acta Derm Venereol (Stockh) 1994; 74: 483-4.

5.2 *In vivo* models for psoriasis

The psoriatic plaque comprises different stages of the psoriatic process in a focal distribution pattern. Hot spots and cold spots or acute and chronic sites exist[1]. Analysis of the initiation and the development of the psoriatic lesion is of pathogenetic relevance. Models for psoriasis might supply a practical tool to study these processes. Also from a therapeutical point of view it is important to develop models for psoriasis. As the therapeutic intervention of relapsing psoriasis might be different from the management of the chronic lesion, it is important to discriminate between models for early or chronic psoriasis. Three models have been investigated in the present thesis:

(1) The response of normal skin to tape stripping (Chapter 2.2).

(2) The response of normal skin to epicutaneous application of leukotriene B_4 (Chapter 3.1).

(3) The relapse of the psoriatic lesion after corticosteroid-induced remission (Chapter 3.2).

TAPE STRIPPING

The epidermal regeneration following tape stripping is a well reproducible response of normal skin which consists of epidermal hyperproliferation and abnormal keratinization as also observed in the psoriatic plaque[2,3]. In Chapter 2.2 it was shown that the three cell biological key features of the psoriatic plaque indeed are observed following tape stripping. The dynamics of the changes proved to be consistent: before the percentage of basal cells in S- and G_2M phase increased, a significant decrease of the number of differentiated cells was observed. This suggests that the suprabasal compartment might have a regulatory role in the initiation of hyperproliferation in the basal compartment.

From a therapeutic point of view it is of relevance that the vitamin D_3 analogue calcipotriol[4], topical corticosteroids[5] and acitretin[6] inhibit epidermal hyperproliferation following tape stripping. Ciclosporin does not exhibit such an effect. The present flow cytometric technique demonstrates a large difference (factor 8.5) between normal skin and tape stripped skin with respect to the proliferative activity of the basal

compartment. Multiparameter flow cytometry promises to be an adequate tool to assess and compare the interference of different therapies with epidermal proliferation, keratinization and inflammation in psoriasis.

LEUKOTRIENE B_4

Cutaneous inflammation is an important aspect of the psoriatic lesion. Leukotriene B_4 (LTB_4) is a potent chemoattractant present in large amounts in psoriatic scales[8]. The response of normal skin to single and multiple applications of LTB_4 is evaluated using multiparameter flow cytometry in Chapter 3.1. This quantitative analysis reveals that single application of LTB_4 induces a cell biological pattern comparable to that of the psoriatic lesion. (an increase of the percentage of cells in S- and G_2M phase, a decrease of the number of differentiated cells and an increase of the number of vimentin-positive cells). In contrast, repeated applications of LTB_4 result in only a minor epidermal hyperproliferation, a decreased number of vimentin-positive cells and an increased number of differentiated cells.
Based on these observations it can be concluded that:

(1) LTB_4 is not relevant in the maintenance of the psoriatic lesion.

(2) LTB_4 might be of pathogenetic importance in the acute stage of the psoriatic process.

RELAPSING PSORIASIS

Perhaps the prevention of the reoccurrence of new lesions is as important as the treatment of existing lesions in the long-term management of psoriasis. However, so far no models to analyse this question are available. By the development of such models new insights in the early stage of psoriasis as well as new information on the effect of treatments of early psoriasis can be obtained. In Chapter 3.2 the relapse following corticosteroid-induced clearance has been characterized both clinically and flow cytometrically. In 80% of patients a relapse occurred within 4 weeks. Occlusion reduced this percentage to 50%. The flow cytometric analysis revealed that symptomless skin located between the relapsing papules already demonstrates a high proliferative activity in the suprabasal compartment, whereas the proliferative activity

in the basal compartment still remains "virtually normal". Therefore it can be concluded that the proliferative activity in the suprabasal compartment is an early phenomenon in the relapse of the psoriatic lesion. It is attractive to speculate that this model will permit studies on the prevention of new lesions by maintenance therapy.

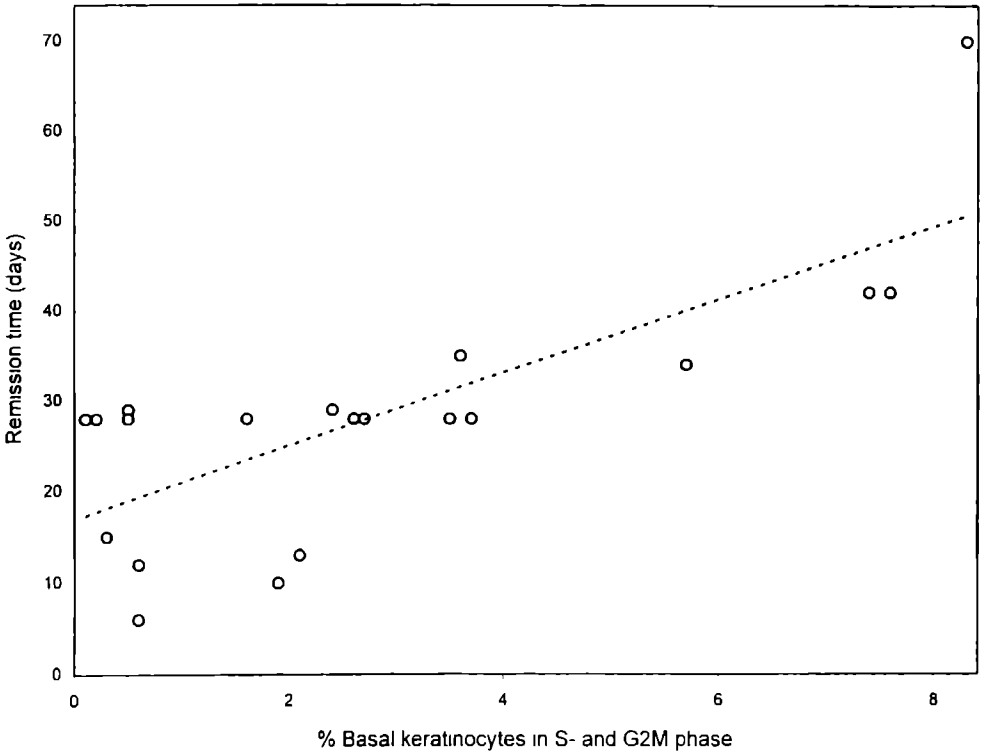


Figure 1. Dot plot of the durations of the remission periods of psoriatic lesions after treatment with clobetasol propionate versus the percentages of basal keratinocytes in S- and G_2M phase (mean ± SEM) at clearance ($r = 0.76$, $P < 0.0001$).

To illustrate and emphasize the specific power of the flow cytometric approach in analysing the relapse of the psoriatic lesion, Figure 1 is introduced. It shows a dot plot of the duration of the remission period following discontinuation of treatment with clobetasol propionate versus the percentages of basal cells in S-and G_2M phases of in total 25 patients (Chapter 3.2 and 4.3). It is evident that those patients with low percentages of basal keratinocytes in S- and G_2M phase at clearance showed a relatively short remission period. In contrast, those patients with higher percentages

had relatively longer remission periods. Decreased proliferative activity seems to imply a quick relapse. One might speculate that these psoriatic lesions were overtreated, resulting in an unstable situation and therefore a short remission time. This phenomenon might be the clue to the rebound effect of intensified topical corticosteroids as observed in some patients.

CONCLUSIONS

(1) Both the tape stripping model as the relapse model suggest that changes in the suprabasal compartment occur well before hyperproliferation of the basal compartment.

(2) Repeated applications of leukotriene B_4 to normal skin result in a cell biological response totally different from the psoriatic lesion. Single application approaches the features of the psoriatic lesion during some days.

(3) Corticosteroid-induced remission constitutes a promising model to evaluate the effect of antipsoriatic drugs on the reappearance of new lesions.

REFERENCES

1 Griffin TD, Lattanand A, VanScott EJ Clinical and histologic heterogeneity of psoriatic plaques Arch Dermatol 1988, 124 216-220.

2 de Mare S, van Erp PEJ, Ramaekers FCS, van de Kerkhof PCM Flow cytometric quantification of human epidermal cells expressing keratin 16 in vivo after standardized trauma Arch Dermatol Res 1990, 282 126-130

3 Gerritsen MJP, van Erp PEJ, van Vlijmen-Willems IMJJ, Lenders LTM et al Repeated tape stripping of normal skin a histological assessment and comparison with events seen in psoriasis Arch Dermatol Res 1994; 286 455-461

4 Arnold WP, van de Kerkhof PCM. The induction of epidermal ornithine decarboxylase following tape stripping is inhibited by a topical vitamin D_3 analogue (MC903) Br J Dermatol 1991, 125 6-8

5. Arnold WP, van Hooijdonk CAEM, Mier PD, van de Kerkhof PCM Induction of ornithine decarboxylase following sellotape stripping in normal and psoriatic skin. Br J Dermatol 1990, 123 581-585

6 Gerritsen MJP, van Pelt HPA, van de Kerkhof PCM Response of the clinically uninvolved skin of psoriatic patients to tape stripping during Acitretin treatment Acta Derm Venereol (Stockh) 1996, 76 6-9

7 Gerritsen MJP, Rulo HFC, Arnold WP, van de Kerkhof PCM Response of the clinically uninvolved skin of psoriatic patients to tape stripping during cyclosporin A treatment Br J Dermatol 1994, 130 181-188

8 Brain SD, Camp RDR, Cunningham FM, Dowd PM et al Leukotriene B_4-like material in scale of psoriatic skin lesions Br J Pharmacol 1984, 83. 313-317

5.3 Innovation of topical antipsoriatic therapy

INTRODUCTION

For the general practitioner or dermatologist who is confronted with a patient suffering from psoriasis different approaches of topical treatment are available (see Chapter 1.1). Tar and dithranol are considered to be effective and safe if the right precautions are taken. However, regimens based on tar or dithranol are often very energy- and time-consuming and therefore usually restricted to in-patient treatment. Topical therapy with corticosteroids combines high efficacy and comfort for the patient. Advantageous is that corticosteroids are easy to apply and that they are odourless and colourless. However, long-term monotherapy with topical corticosteroids induces skin atrophy and striae. Therefore, new antipsoriatic strategies are needed.

In recent years, topical treatment of psoriasis has been developed in two directions. Firstly, the introduction of the vitamin D_3 analogues induced an expansion of the therapeutic arsenal against psoriasis. Secondly, the principle of hydrocolloid occlusion in combination with corticosteroids meant an important progress.

In the following sections these issues are discussed.

VITAMIN D_3 ANALOGUES

In vitro data demonstrate the interference of several topical vitamin D_3 derivatives with various characteristics of the psoriatic plaque (see Chapter 4.1 and 4.2). During the last decade the clinical efficacy of calcitriol (1α,25 dihydroxyvitamin D_3), calcipotriol and Tacalcitol (1α,24 dihydroxyvitamin D_3) has been documented[1-3]. In comparison to calcitriol, both calcipotriol and Tacalcitol exert a decreased calcitropic effect[4,5]. Calcipotriol monotherapy (50μg/g ointment twice daily) has been shown to be at least equally effective as a third-class corticosteroid[6,7]. Comparative studies demonstrated that calcipotriol was also more effective than tar therapy[8] or short-contact dithranol therapy[9]. However, an important limitation is the calcipotriol-induced irritative dermatitis that occurs in up to 25% of patients[10].

One way to reduce the frequency of this dermatitis could be the combination of calcipotriol (the only registered vitamin D_3 analogue in the Netherlands) with topical corticosteroids. Another way could be to reduce the calcipotriol application to only once daily. A third way could be the development of vitamin D_3 analogues with less

irritative potential. Remarkably, with respect to Tacalcitol (2μg/g ointment twice daily) only a slight irritation was reported in less than 1% of Japanese patients[4].
Therefore we were interested to establish both the quantitative efficacy and the tolerability of (1) calcipotriol monotherapy versus the combination of calcipotriol and corticosteroids (Chapter 4.1) and (2) antipsoriatic therapy with Tacalcitol (Chapter 4.2).
The conclusions are as follows:

(1) Vitamin D_3 analogues seem to exert their *in vivo* antipsoriatic effect mainly through an inhibition of epidermal growth.

(2) The cell biological effect of calcipotriol applied once daily is inferior to calcipotriol twice daily. This effect was also reflected clinically in a large multicentre comparative study[11].

(3) The antiproliferative potential of Tacalcitol (once daily) and calcipotriol (twice daily) is comparable, although the clinical efficacy of Tacalcitol tended to be slightly inferior; so far no comparative clinical studies are available.

(4) Concurrent use of calcipotriol and a topical corticosteroid has a marked antiinflammatory effect compared to calcipotriol monotherapy.

(5) Combination of calcipotriol and a topical corticosteroid reduces the frequency of irritative dermatitis compared to calcipotriol monotherapy.

(6) The low irritative potential of Tacalcitol permits treatment of facial and flexural psoriatic lesions (see Fig. 1)[12].

Figure 1. Facial psoriatic lesion before (a) and after 8 weeks treatment (b) with Tacalcitol (1α,24 dihydroxyvitamin D_3) 4μg/g once daily. During treatment the clinical signs of the lesion almost totally disappeared and no irritation developed.

COMBINATION OF TOPICAL CORTICOSTEROIDS AND HYDROCOLLOID OCCLUSION

In the treatment of recalcitrant psoriatic plaques the use of strong antipsoriatic corticosteroids is inevitable. The total exposure to corticosteroids might be reduced by using these agents in combination with hydrocolloid occlusion. Various studies demonstrated the therapeutic effect of hydrocolloid occlusive dressings alone on psoriasis[13-15]. It has been shown that a strong clinical response can be obtained with topical corticosteroids in combination with hydrocolloid occlusion (see Chapter 4.3).

However, comparative clinical and flow cytometric studies between steroid monotherapy and the above-mentioned combination therapy with respect to clearance and relapse have not been carried out so far.
The conclusions with respect to these issues can be summarized as follows:

(1) Both corticosteroid monotherapy twice daily and topical corticosteroids once weekly under hydrocolloid occlusion exert a strong and comparable antipsoriatic efficacy with respect to both clinical and cell biological parameters.

(2) Clearance of psoriatic plaques seems to be induced earlier with the combination.

(3) With respect to the relapse phase no difference in clinical and cell biological parameters between both treatments can be demonstrated.

Hydrocolloid occlusion is a valuable approach to enhance bioavailability of topical drugs. It is attractive to speculate that other topical treatments might benefit from hydrocolloid occlusion.

REFERENCES

1 Langner A, Verjans H, Stapor V, Mol M et al Topical calcitriol in the treatment of chronic plaque psoriasis a double-blind study Br J Dermatol 1993, 128 566-571

2 Kragballe K, Beck HI, Sogaard H. Improvement of psoriasis by a topical vitamin D3 analogue (MC 903) in a double-blind study Br J Dermatol 1988, 119 223-230

3 Kato T, Rokugo M, Terui T, Tagami H Successful treatment of psoriasis with topical application of active vitamin D3 analogue, 1 alpha,24-dihydroxycholecalciferol Br J Dermatol 1986, 115 431-433

4 Nishimura M, Hori Y, Nishiyama S, Nakamizo Y Topical 1a,24 (R)-dihydroxyvitamin D_3 for the treatment of psoriasis. Review of the literature Eur J Dermatol 1993, 3 255-261.

5 Matsunaga T, Yamamoto M, Mimura H, Ohta T et al. 1,24(R)-dihydroxyvitamin D3, a novel active form of vitamin D3 with high activity for inducing epidermal differentiation but decreased hypercalcemic activity J Dermatol 1990, 17 135-142

6 Kragballe K, Gjertsen BT, de Hoop D, Karlsmark T et al Double-blind, right/left comparison of calcipotriol and betamethasone valerate in treatment of psoriasis vulgaris Lancet 1991, 337 193-196

7 Cunliffe WJ, Berth-Jones J, Claudy A, Fairiss G et al. Comparative study of calcipotriol (MC 903) ointment and betamethasone 17-valerate ointment in patients with psoriasis vulgaris. J Am Acad Dermatol 1992; 26: 736-743.

8 Tham SN, Lun KC, Cheong WK. A comparative study of calcipotriol ointment and tar in chronic plaque psoriasis. Br J Dermatol 1994; 131: 673-677.

9 Berth-Jones J, Chu AC, Dodd WAH, Ganpulle M et al. A multicentre, parallel-group comparison of calcipotriol ointment and short-contact dithranol therapy in chronic plaque psoriasis. Br J Dermatol 1992; 127. 266-271.

10 Ramsay CA, Berth-Jones J, Brundin G, Cunliffe WJ et al. Long-term use of topical calcipotriol in chronic plaque psoriasis. Dermatology 1994; 189: 260-264.

11 Griffin TD, Lattanand A, VanScott EJ. Clinical and histologic heterogeneity of psoriatic plaques. Arch Dermatol 1988; 124: 216-220.

12 van de Kerkhof PCM. Calcipotriol cream and concurrent corticosteroids in psoriasis. JEADV 1996; Suppl. 1: s184.

13 Friedman SJ. Management of psoriasis vulgaris with a hydrocolloid occlusive dressing. Arch Dermatol 1987; 123: 1046-1052.

14 Gerritsen MJP, van Vlijmen-Willems IMJJ, Chang A, van de Kerkhof PCM. The effect of a hydrocolloid occlusive dressing (DuoDERM E) on keratinization in psoriasis vulgaris. Acta Derm Venereol (Stockh) 1994; 74: 483-484.

15 Telfer NR, Ryan TJ, Blanc D, Merk H et al. Results of a multicentre trial of actiderm in the treatment of of plaque psoriasis. In: Beyond occlusion: dermatology proceedings (Ryan TJ, ed), London, New York:Royal Society of Medicine Services Limited, 1988; 53-58.

Chapter 6

SUMMARY AND CONCLUSIONS
SAMENVATTING EN CONCLUSIES
DANKWOORD
CURRICULUM VITAE
LIST OF PUBLICATIONS

Summary and Conclusions

In the first part of Chapter 1 a general review on the epidemiology, the pathogenesis and the morphology of psoriasis is given Several antipsoriatic therapeutic modalities are described Furthermore, the clinical assessment of the severity of the psoriatic lesion is discussed This assessment is subjective and therefore inaccurate Clearly, a need for quantitative analysis of disease activity exists In the second part of Chapter 1 a short review on the history of flow cytometry is presented It is explained why multiparameter flow cytometry seems to be an attractive tool to quantify aspects of epidermal proliferation, keratinization and cutaneous inflammation of the normal and pathological skin In the last part of Chapter 1 the three major aims of the present thesis are presented

(1) To develop and validate a new approach to monitor disease activity This new method should permit quantitative measurement and reflect the key processes in the pathogenesis of psoriasis

(2) To characterize potential *in vivo* models for psoriasis

(3) To develop and evaluate new strategies in the topical treatment of psoriasis

In Chapter 2 methodological aspects of multiparameter flow cytometry are described The new DNA fluorochrome TO-PRO-3 iodide is introduced This HeNe laser-excited fluorochrome proves to be an excellent alternative for propidium iodide in multiparameter flow cytometry An important advantage of TO-PRO-3 iodide is the relatively small spectral overlap with phycoerythrin This means that only a minor electronic compensation for this overlap is needed, resulting in optimal measurements High-quality cell suspensions are essential in flow cytometry of epidermal cells Development and validation of a new dermo-epidermal separation method using the enzyme thermolysin are described In contrast to separation with only trypsin this method is also applicable on punch biopsies

Multiparameter flow cytometry proves to be an optimal approach to analyse epidermal single-cell suspensions from normal and hyperproliferative skin Epidermal

subpopulations can be discriminated and reliable cell cycle analysis can be performed. Quantitative evaluation of the tape stripping model reveals that the proliferative activity in the basal compartment is increased by a factor 8.5 during epidermal regeneration after standardized injury.
It is concluded that multiparameter flow cytometry promises to be an adequate tool to evaluate antipsoriatic therapy.

In Chapter 3 two other *in vivo* models for psoriasis are investigated: (1) single and multiple applications of leukotriene B_4 to normal skin and (2) the relapse of psoriatic skin after treatment with topical corticosteroids under occlusion. Flow cytometric analysis shows that the effect of chronic applications of leukotriene B_4 to normal skin is markedly different from the cell biological features of the psoriatic plaque. In contrast, single challenge produces changes comparable to psoriasis. Therefore, leukotriene B_4 does not seem of great importance for perpetuation of the chronic psoriatic plaque, but may play a role in the initiation of the psoriatic lesion.
Clinical and flow cytometric analysis shows that the third *in vivo* model (relapse of psoriatic skin after clearance) is an adequate model to study early events in the psoriatic lesion. Furthermore, this model allows standardized comparison of different approaches for maintenance therapy in psoriasis. It is demonstrated that the proliferative activity in the suprabasal compartment seems to be an early characteristic of relapse after clinical clearance of the psoriatic lesion.

Chapter 4 describes the effects of the vitamin D_3 analogue 1,24 dihydroxyvitamin D_3 (Tacalcitol), the effects of different combinations of calcipotriol (Daivonex) and topical corticosteroids, and the effects of corticosteroid monotherapy (with or without hydrocolloid occlusion) on epidermal growth, keratinization and inflammation. Vitamin D_3 analogues seem to exert their antipsoriatic effect mainly through an inhibition of epidermal growth. In contrast, topical corticosteroids (monotherapy or combination therapy) have a marked antiinflammatory effect which is clinically expressed by the erythema score. The combination of calcipotriol and corticosteroids proves to have a strong antipsoriatic potential. With respect to relapse characteristics the use of topical corticosteroids under hydrocolloid occlusion is comparable with the classical corticosteroid treatment.

In Chapter 5 the results of the various investigations of the present thesis are summarized and discussed. With respect to the aims of the thesis the following conclusions are formulated:

(1) Multiparameter flow cytometry is an accurate and attractive tool to monitor psoriatic disease activitity. Strong correlations exist between clinical and flow cytometric parameters, especially between the induration score and the proliferative activity of the basal compartment.

(2) Multiparameter flow cytometry permits quantitative characterization and evaluation of *in vivo* models for psoriasis. Single application of leukotriene B_4 to normal skin and the relapsing psoriatic skin following topical steroid treatment are adequate models for early psoriasis.

(3) New developments in topical treatment of psoriasis are promising. Multiparameter flow cytometry is an adequate tool to quantify and compare the cell biological effects of these new antipsoriatic strategies.

Samenvatting en Conclusies

In het eerste deel van Hoofdstuk 1 wordt een algemeen overzicht gegeven van de epidemiologie, de pathogenese en de morfologie van psoriasis Meerdere antipsoriatische behandelingsmodaliteiten worden beschreven Daarnaast wordt de klinische bepaling van de ernst van een psoriatische laesie besproken Deze bepaling is subjectief en inaccuraat Derhalve bestaat er een duidelijke behoefte naar quantitatieve analyse van ziekteactiviteit In het tweede deel van Hoofdstuk 1 wordt in een kort overzicht de geschiedenis van de flowcytometrie geschetst Aangegeven wordt waarom multiparameter flowcytometrie een zo aantrekkelijk instrument is om aspecten van epidermale groei, keratinisatie en cutane inflammatie te quantificeren in de normale en pathologisch veranderde huid In het laatste deel van Hoofdstuk 1 worden de drie doelstellingen van deze thesis gepresenteerd

(1) De ontwikkeling en validering van een nieuwe methode om ziekteactiviteit te vervolgen Voorwaarden hierbij zijn de mogelijkheid tot quantitatieve bepalingen en de weerspiegeling van de elementaire processen in de pathogenese van psoriasis

(2) De karakterisering van potentiele *in vivo* modellen voor psoriasis

(3) De ontwikkeling en evaluatie van nieuwe lokale behandelingsmodaliteiten voor psoriasis

Hoofdstuk 2 beschrijft methodologische aspecten van de multiparameter flowcytometrie Het nieuwe DNA-fluorochroom TO-PRO-3 jodide wordt geintroduceerd Dit door de HeNe-laser geexciteerde fluorochroom blijkt in de multiparameter flowcytometrie een uitstekend alternatief te zijn voor propidiumjodide Een belangrijk voordeel van TO-PRO-3 jodide is de relatief geringe spectrale overlap met phycoerythrine Dit betekent dat voor deze overlap nauwelijks elektronische compensatie nodig is Dit resulteert in optimale metingen
Celsuspensies van hoge kwaliteit zijn essentieel voor flowcytometrie van epidermale cellen De ontwikkeling en validering van een nieuwe dermo-epidermale scheidingsmethode met het enzym thermolysine wordt beschreven In tegenstelling tot

scheiding met alleen trypsine kan deze methode ook toegepast worden op punchbiopten.
Multiparameter flowcytometrie blijkt een optimale methode te zijn ter analyse van epidermale celsuspensies van normale en hyperproliferatieve huid. Meerdere epidermale subpopulaties kunnen onderscheiden worden en betrouwbare celcyclusanalyse kan uitgevoerd worden. Kwantitatieve analyse van het tape-stripping-model laat zien dat tijdens de epidermale regeneratie na gestandaardiseerd trauma de proliferatieve activiteit in het basale compartiment toeneemt met een factor 8,5.
We concluderen dat multiparameter flowcytometrie een veelbelovend instrument lijkt te zijn ter evaluatie van antipsoriatische therapieën.

In Hoofdstuk 3 worden twee andere *in vivo*-modellen voor psoriasis bestudeerd: (1) éénmalige en multiple applicatie van leukotriëne B_4 op de normale huid en (2) de relapse van psoriatische huid na behandeling met lokale corticosteroïden onder occlusie. Flowcytometrische analyse toont aan dat het effect van chronische applicaties van leukotriëne B_4 op de normale huid duidelijk verschilt van de celbiologische kenmerken van de psoriatische plaque. Eénmalige applicatie, daarentegen, resulteert in met psoriasis vergelijkbare veranderingen. Dus lijkt leukotriëne B_4 niet van groot belang voor de instandhouding van de chronische psoriatische plaque, maar speelt mogelijk een rol bij de initiatie van de psoriatische laesie.
Klinische en flowcytometrische analyse van het derde *in vivo*-model (relapse van psoriatische huid na clearance) demonstreert dat dit een adequaat model is ter bestudering van vroege processen in de psoriatische laesie. Daarnaast maakt dit model een gestandaardiseerde vergelijking van verschillende methoden van onderhoudstherapie bij psoriasis mogelijk. De relapse van de psoriatische laesie na klinische clearance wordt gekarakteriseerd door met name proliferatieve activiteit in het suprabasale compartiment.

Hoofdstuk 4 beschrijft de effecten van vitamine D_3-derivaat 1,24 dihydroxyvitamine D_3 (Tacalcitol), de effecten van verschillende combinaties van calcipotriol (Daivonex) en lokale corticosteroïden en de effecten van corticosteroïd-monotherapie (met of zonder hydrocolloïd-occlusie) op epidermale groei, keratinisatie en inflammatie. De antipsoriatische werking van vitamine D_3-derivaten berust met name op een inhibitie

van epidermale groei. Lokale corticosteroïden, daarentegen, bezitten bij zowel mono- als combinatietherapie een uitgesproken anti-inflammatoir effect. Dit wordt klinisch tot uiting gebracht in de erythema-score. De combinatie van calcipotriol met corticosteroïden heeft een sterke antipsoriatische werking. Wat betreft relapse-karakteristieken is het gebruik van corticosteroïden onder hydrocolloïd-occlusie geheel vergelijkbaar met klassiek corticosteroïdgebruik.

In Hoofdstuk 5 worden de resultaten van de verschillende studies van deze thesis samengevat en besproken. Met betrekking tot de doelstellingen kunnen de volgende conclusies geformuleerd worden:

(1) Multiparameter flowcytometrie is een accurate and attractieve methode om psoriatische ziekteactiviteit te vervolgen. Er bestaat een sterke correlatie tussen klinische en flowcytometrische parameters, met name tussen de induratie-score en de proliferatieve activiteit van het basale compartiment.

(2) Multiparameter flowcytometrie bewerkstelligt een quantitatieve karakterisatie en evaluatie van *in vivo*-modellen voor psoriasis. De éénmalige applicatie van leukotriëne B_4 op normale huid en de relapse van psoriatische huid na lokale behandeling met corticosteroïden zijn geschikte modellen voor vroege vormen van psoriasis.

(3) Nieuwe ontwikkelingen in de lokale behandeling van psoriasis zijn veelbelovend. Multiparameter flowcytometrie is een zeer geschikt instrument om de celbiologische effecten van nieuwe antipsoriatische strategieën te quantificeren en te vergelijken.

Dankwoord

Graag wil ik allen die bijgedragen hebben aan het tot stand komen van dit proefschrift zeer hartelijk bedanken. Een promotie-onderzoek kan alleen dan tot stand komen, als de kunde en inspanningen van vele personen gebundeld worden. Het is in dit korte bestek niet mogelijk iedereen te vermelden, maar enkele personen wil ik graag persoonlijk noemen.

Als eerste, natuurlijk, mijn promotor Prof. Dr. Dr. P.C.M. van de Kerkhof. Peter, jij bent degene die mij binnengehaald heeft op de afdeling Dermatologie en die het mij mogelijk maakte mijn carrière in de dermatologie te beginnen. Jouw inspiratie, drive en enthousiasme hebben in belangrijke mate de omstandigheden geboden, waarin met plezier efficiënt gewerkt kan worden. Altijd was jij zeer stimulerend aanwezig en bereid om de voortgang van het onderzoek te bespreken en resultaten te bediscussiëren. Als geen ander besef jij dat de combinatie van een goede sfeer en de innige samenwerking tussen laboratorium, polikliniek en kliniek voorwaarden zijn voor creatief wetenschappelijk en klinisch werk.

Uiteraard wil ik ook mijn co-promotoren Dr. P.E.J. van Erp en Dr. E.M.G.J de Jong noemen. Piet, met jou heb ik de methodologische basis van dit proefschrift kunnen leggen. Jouw grote kennis van de flowcytometer als technisch apparaat was onmisbaar voor dit onderzoek. Nog goed herinner ik me de lange middagen en avonden waarop we de triple-labelling ontwikkelden. Jij hebt me ingewijd in de geheimen van de flowcytometrie en me bijgestaan met je goede raad en praktische adviezen. Elke, bedankt voor je hulp, met name bij de afronding van het manuscript.

Pipetteren, celsuspensies maken, nauwkeurig wegen, flowcytometrische protocollen, etc., etc., etc. Van wie kun je het beter leren dan van Candida van Hooijdonk? Candida, bedankt voor je volhardende inspanningen om van een arts een labmedewerker te maken. Het zal me bijblijven.

René Botermans, Etienne Meulen en Bianca Seegers wil ik danken voor hun bijdragen binnen het kader van hun wetenschappelijke stages. Jullie begeleiden heeft mij veel geleerd. Gijs de Jongh en Jan Boezeman wil ik graag danken voor hun bijstand tijdens mijn statistische exercities. Natuurlijk ben ik grote dank verschuldigd aan alle patiënten die hun medewerking aan de verschillende studies verleend hebben.

Als laatsten wil ik Wilma, Laura en Thomas danken voor hun opgewekte, steunende en liefhebbende aanwezigheid in mijn leven.

Curriculum Vitae

De schrijver van dit proefschrift werd op 17 november 1963 in Heidelberg (Duitsland) geboren. In 1982 behaalde hij het einddiploma Gymnasium β aan het Stedelijk Lyceum te Zutphen. In datzelfde jaar begon hij met de studie geneeskunde aan de Katholieke Universiteit Nijmegen. Het artsexamen werd afgelegd in september 1991.
Van maart 1992 tot februari 1993 vervulde hij een assistentschap chirurgie in het Ziekenhuiscentrum te Apeldoorn, waarna een korte periode als arts-assistent dermatologie in het Elisabeth Gasthuis te Haarlem volgde. Sinds juni 1993 is hij verbonden aan de afdeling dermatologie van het Academisch Ziekenhuis Nijmegen, waar onder leiding van promotor Prof. Dr. Dr. P.C.M. van de Kerkhof de werkzaamheden aan dit proefschrift gestart werden. Sinds maart 1996 is hij in opleiding tot dermatoloog in het Academisch Ziekenhuis te Nijmegen (opleider: Prof. Dr. Dr. P.C.M. van de Kerkhof).
Hij is getrouwd met Wilma Veldhorst en de trotse vader van Laura en Thomas.

List of Publications

1. Arnold WP, Glade CP, Mier PD, van de Kerkhof PCM. Effects of sphingosine, isoquinoline and tannic acid on the human tape-stripping model and the psoriatic lesion. Skin Pharmacology 1993; 6: 193-199.
2. van Hooijdonk CAEM, Glade CP, van Erp PEJ. TO-PRO-3 iodide: a novel HeNe laser-excitable DNA stain as an alternative for propidium iodide in multiparameter flow cytometry. Cytometry 1994; 17: 185-189.
3. Glade CP, van Erp PEJ, van Hooijdonk CAEM, Elbers ME, van de Kerkhof PCM. Topical treatment of psoriatic plaques with 1α,24 dihydroxyvitamin D3: a flow cytometrical multiparameter analysis of epidermal growth, differentiation and inflammation. Acta Derm Venereol (Stockh) 1995; 75: 381-385.
4. Glade CP, Botermans RJG, van Erp PEJ, van de Kerkhof PCM. The dynamics of the response of normal skin to single and multiple epicutaneous leukotriene B_4 applications analysed by three-colour flow cytometry. Acta Derm Venereol (Stockh) 1995; 75: 437-441.
5. Glade CP, Seegers BAMPA, Meulen EFJ, van Hooijdonk CAEM, van Erp PEJ, van de Kerkhof PCM. Multiparameter flow cytometric characterization of epidermal cell suspensions prepared from normal and hyperproliferative skin using an optimized thermolysin-trypsin protocol. Arch Dermatol Res 1996; 288: 203-210.
6. Epidermal cell DNA content and intermediate filaments keratin 10 and vimentin after treatment of psoriatic plaques with calcipotriol cream once daily, twice daily and in combination with clobetasone 17-butyrate cream or betamethasone 17-valerate: a comparative flow cytometric study. Br J Dermatol 1996; 135: 379-384.
7. Glade CP, van der Vleuten CJM, van Erp PEJ, van de Kerkhof PCM. Flow cytometric assessment of clearance and relapse characteristics in psoriasis vulgaris after treatment with weekly clobetasol lotion under occlusion versus twice daily clobetasol ointment (to be submitted).
8. Glade CP, van Erp PEJ, Werner-Schlenzka H, van de Kerkhof PCM. A clinical and flow cytometric validation of a model to study remission and relapse in psoriasis. Acta Derm Venereol (Stockh) (in press).
9. Glade CP, van Erp PEJ, Boezeman JBM, van de Kerkhof PCM. Multiparameter flow cytometry as a tool to evaluate antipsoriatic therapy. Br J Dermatol (in press).

10. Glade CP, van der Vleuten CJM, van Erp PEJ, de Jong EMGJ, van de Kerkhof PCM. The epidermis of chronic idiopathic lichen planus during topical treatment with the vitamin D_3 analogue KH 1060 (submitted).
11. Seegers BAMPA, Glade CP, van Hooijdonk CAEM, van Erp PEJ, van de Kerkhof PCM. Flow cytometric characterization of normal versus psoriatic epidermis using an improved cell separation methodology (submitted).

STELLINGEN

behorende bij het proefschrift

MULTIPARAMETER FLOW CYTOMETRY AS A TOOL TO STUDY PSORIASIS

Conrad P. Glade, Nijmegen, 9 juni 1997

Het fluorochroom TO-PRO-3 jodide is een uitstekend alternatief voor propidiumjodide bij de bepaling van relatieve DNA hoeveelheid en is gezien de geringe spectrale overlap met phycoerythrine uitermate geschikt voor multiparameter flowcytometrie.
-dit proefschrift-

Het enzym thermolysine bewerkstelligt een betrouwbare dermo-epidermale separatie.
-dit proefschrift-

Leukotriëne B_4 speelt geen grote rol bij de instandhouding van de chronische psoriatische plaque.
-dit proefschrift-

De relapse na clearance van psoriatische huid is een geschikt *in vivo*-model ter bestudering van vroege processen in de psoriatische laesie.
-dit proefschrift-

In vivo oefenen de vitamine D_3 derivaten calcipotriol en 1α,24-dihydroxyvitamine D_3 hun antipsoriatische werking met name uit door remming van epidermale groei.
-dit proefschrift-

Bij de evaluatie van antipsoriatische therapieën is de multiparameter flowcytometrie een waardevolle aanvulling op de "Psoriasis Area and Severity Index (PASI).
-dit proefschrift-

A 51 μm particle clogs a 50 μm orifice.
-Shapiro's first law of flow cytometry-

Een instrument kun je niet dwingen, maar moet je laten spelen. Dit geldt niet alleen voor mijn cello.

No data analysis technique can make good data out of bad data.
-Shapiro's seventh law of flow cytometry-

Binnen de dermatologische deelgebieden begint in Nijmegen de dermatopatisserie steeds vastere vorm aan te nemen.

Alleen al de irritatie over het verstrekken van taxibriefjes betekent een grote belasting voor de gezondheid.